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(54) Title: LIVE ATTENUATED VIRUS VACCINES FOR EQUINE ENCEPHALITIS VIRUSES

(57) Abstract

cDNAs coding for an infectious Western Equine Encephalitis virus (WEE) and infectious Venezuelan Equine Encephalitis virus variant IE (VEE IE) are disclosed in addition to cDNA coding for the structural proteins of Venezuelan Equine Encephalitis virus variant IIIA (VEE IIIA). Novel attenuating mutations of WEE and VEE IE and their uses are described. Also disclosed are attenuated chimearic alphaviruses and their uses.

TITLE OF THE INVENTION

LIVE ATTENUATED VIRUS VACCINES FOR EQUINE ENCEPHALITIS VIRUSES

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INTRODUCTION

Western equine encephalitis (WEE), eastern equine
15 encephalitis (EEE) and Venezuelan equine encephalitis
virus (VEE) are members of the alphavirus genus of the
family Togaviridae which is comprised of a large group
of mosquito-borne RNA viruses found throughout much of
the world. The viruses normally circulate among
20 rodent or avian hosts through the feeding activities
of a variety of mosquitoes. Epizootics occur largely
as a result of increased mosquito activity after
periods of increased rainfall. Western equine
encephalitis virus (WEE) was first recognized in 1930
25 and causes periodic outbreaks of disease in equines.
The virus has been detected over much of the western
hemisphere from Argentina north to the more temperate
regions of central Canada (For a review, see Reisen
and Monath [1988] in The Arboviruses: Epidemiology and
30 Ecology, Vol. V. CRC Press, Inc. Boca Raton).
Similarly, EEE was first isolated in Virginia and New
Jersey in 1933 (Ten Broeck, C. et al. [1935] J. Exp.
Med. 62:677) and is now known to be focally endemic
throughout much of the northern portion of South

America, Central America and the eastern part of
Mexico and the United States. Venezuelan equine
encephalitis virus has six serological subtypes (I-
VI). Two of these subtypes, I and III have multiple
5   variants, two of these variants are of particular
interest in this application, variant IE, and variant
IIIA also called Mucambo virus. A live, attenuated
vaccine (TC-83) for VEE IA/B has been used for
immunization equines and at-risk laboratory and field
10   personnel (Birge et al. [1961] Am. J. Hyg. **73**:209-218;
Pittman et al. [1996] Vaccine **14**:337-343). The
vaccine was credited with helping to limit the
northward spread of a serious epizootic of VEE
originating in South America in the late 1960's.
15   However, the VEE I/AB vaccines have not yet been
licensed by the Food and Drug Administration and have
been shown to be effective in preventing disease from
VEE IA/B infection only. The current VEE vaccines do
not adequately protect against the VEE IE variant or
20   the VEE IIIA variant, as disease has occurred in
laboratory workers successfully vaccinated with a
vaccine derived from VEE IA/B. In addition, recent
unprecedented outbreaks of VEE IE in populations of
horses in Mexico indicate a need for a VEE IE vaccine.
25   The lack of adequate cross protection with existing
IA/B vaccines documents the need for a VEE IE-specific
and a VEE IIIA-specific vaccine.
        The vaccines currently in veterinary use for WEE,
EEE and VEE IA/B throughout the United States and
30   Canada are formalin-inactivated preparations.
Inactivated vaccines for EEE and WEE are also
available for use by at-risk laboratory personnel.
These inactivated vaccines are poorly immunogenic,
require multiple inoculations with frequent boosters
35   and generally result in immunity of short duration.

The shortcomings of the available vaccines indicate a
need for the development of new vaccines of high
immunogenicity which induce a longer lasting immunity
for protection against WEE, EEE and VEE subtypes IE
5   and IIIA.

SUMMARY OF THE INVENTION
The present invention satisfies the need
mentioned above.
10   In this application are described live attenuated
vaccines for WEE, EEE, VEE IE and VEE IIIA which may
provide higher level immunity in humans and equines
for many years, and possibly for life. In addition,
very large numbers of vaccine doses can be produced
15   from significantly less starting materials than is
possible with the existing inactivated products. The
vaccine preparations of the present invention comprise
full-length cDNA copies of the genomes of WEE or VEE
IE which have been altered such that the RNA produced
20   from the cDNA, and the virus produced therefrom is
attenuated and useful as a live vaccine for human and
veterinary use. The vaccine preparations for VEE IIIA
and EEE are novel chimeric viruses which include the
newly discovered structural protein genes of VEE IIIA.
25   The classic methods of deriving live-attenuated
vaccines (blind passage in cell cultures) generally
result in heterogeneous and undefined products, hence
recent attempts to make live vaccines for alphaviruses
have relied on genetic engineering procedures.
30   The alphavirus genome is a single-stranded,
positive-stranded RNA approximately 11,400 nucleotides
in length. The 5' two-thirds of the genome consist of
a non-coding region of approximately 48 nucleotides
followed by a single open reading frame of
35   approximately 7,500 nucleotides which encodes the

viral replicase/transcriptase. The 3' one-third of
the genome encodes the viral structural proteins in
the order C-E3-E2-6K-E1, each of which are derived by
proteolytic cleavage of the product of a single open
5   reading frame of approximately 3700 nucleotides. The
sequences encoding the structural proteins are
transcribed as a 26S mRNA from an internal promoter on
the negative sense complement of the viral genome.
The nucleocapsid (C) protein possesses autoproteolytic
10   activity which cleaves the C protein from the
precursor protein soon after the ribosome transits the
junction between the C and E3 protein coding sequence.
Subsequently, the envelope glycoproteins E2 and E1 are
derived by proteolytic cleavage in association with
15   intracellular membranes and form heterodimers. E2
initially appears in the infected cell as a
precursor, pE2, which consists of E3 and E2. After
extensive glycosylation and transit through the
endoplasmic reticulum and the golgi apparatus, E3 is
20   cleaved from E2 by furin-like protease activity at a
cleavage site having a consensus sequence of RX(K/R)R,
with X being one of many amino acids present in the
different viruses, and with the cleavage occuring
after the last arginine residue. Subsequently, the
25   E2/E1 complex is transported to the cell surface where
it is incorporated into virus budding from the plasma
membrane (Strauss and Strauss [1994] *Microbiological
Rev.* **58**: 491-562). All documents cited herein *supra*
and *infra* are hereby incorporated in their entirety by
30   reference thereto.

    Because the genome of alphavirus is a positive-
stranded RNA, and infectious upon transfection of
cells in culture, an "infectious clone" approach to
vaccine development is particularly suitable for the
35   alphaviruses. In this approach, a full-length cDNA

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clone of the viral genome is constructed downstream from a RNA polymerase promoter, such that RNA which is equivalent to the viral genome can be transcribed from the DNA clone *in vitro*. This allows site-directed
5   mutagenesis procedures to be used to insert specific mutations into the DNA clone, which are then reflected in the virus which is recovered by transfection of the RNA.

Previous work with infectious clones of other
10 alphaviruses has demonstrated that disruption of the furin cleavage site results in a virus which incorporates pE2 into the mature virus. Davis *et al.* (1995, *supra*) found that disruption of the furin cleavage site in an infectious clone of VEE is a
15 lethal mutation. Transfection of BHK cells with RNA transcribed from this mutant clone resulted in the release of non-infectious particles. However, a low level of infectious virus was produced which contained secondary suppressor mutations such that virus
20 containing pE2 was fully replication competent and subsequently shown to be avirulent but capable of elliciting immunity to lethal virus challenge in a variety of animal species.

The genetic basis for attenuation of the VEE TC-
25 83 vaccine and certain laboratory strains of VEE virus have been studied extensively and has led to the development of improved live, attenuated vaccine candidates (Kinney *et al.* 1993, *supra*, Davis *et al.* 1995, *supra*). The approach used in this application
30 is similar to that used for VEE, however, following the VEE example exactly did not result in an adequate vaccine for WEE. Changes in the procedure used for VEE were required, none of which could have been predicted from the VEE work, in order to produce the
35 attenuated live WEE virus of the present invention.

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Based upon a comparison of the structural protein gene sequences of WEE and other alphaviruses, the probable furin cleavage site of WEE strain CBA/87 virus is RRPKR. The presence of the extra arginine
5   when compared to the conscensus (RX(R/K)R) alphavirus cleavage site indicated that the cleavage at this site might be more complex than that observed with VEE virus. It was necessary therefore to prepare two deletion mutations in the E3-E2 cleavage site of the
10 full-length clone, one which lacks five amino acids and one which lacks four amino acids since it was unknown which mutation, if any, would produce an attenuated virus. The residual arginine in the full-length clone lacking only four amino acids was of
15 concern due to the possibility that other mutations might arise due to the presence of the extra arginine resulting in cleavage by cellular proteases at that site and producing an apparently wild type virus with respect to cleavage of pE2.
20   Transfection of cultured cells with RNA transcribed from an infectious clone of WEE lacking the furin cleavage site yielded viruses which contained the pE2 of WEE in the mature virus but which were not replication competent. During intracellular
25 replication of the RNA, mutations arise at low frequency, resulting in a small number of replication competent virus. Sequence analysis of these viruses has shown that the lethal effect of the deletion mutations was alleviated by the appearance of second
30 site mutations in the E2 glycoprotein. These viruses are attenuated in mice when administered by subcutaneous or intracranial inoculation. The mice produce high titer neutralizing and ELISA antibody and are protected against a lethal challenge of parental
35 virulent WEE virus.

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Therefore, in one aspect of the invention, the invention pertains to the isolation of a cDNA sequence coding for an infectious western equine encephalitis (WEE) virus RNA transcript. DNA representing the

5   entire genome, not previously available, was prepared by polymerase chain reaction using a series of primer pairs based upon the partial genome sequences previously deposited in Genbank. The 5' and 3' ends of the viral genome were unknown and difficult to

10   obtain. The terminal sequence was necessary for efficient replication of the virus since substitution of ends from a similar virus with similar but not identical sequences resulted in an extremely attenuated virus. In order to determine the correct

15   sequence at the 5' end, a protocol called rapid amplification of cDNA ends (5'-RACE) was used. The full length infectious clone is useful in the production of virulent WEE virus, and introducing and testing attenuating mutations. The production of

20   virulent virus is essential for a formal measure of the degree of attenuation achieved with candidate attenuating mutations and a formal determination of the rate at which reversion to virulence might occur.

        In another aspect of the invention, the invention

25   pertains to the isolation of a cDNA sequence coding for an infectious Venezuelan equine encephalitis virus IE variant (VEE IE) virus RNA transcript (SEQ ID NO: 2). Using oligonucleotides specific to genomic RNA of a VEE IE isolate (GenBank accession no. U34999)

30   (Oberste, *et al.* [1996] *Virology* **219**:314-320), reverse transcriptase polymerase chain reaction was carried out to generate numerous cDNA fragments which were subsequently cloned and used to assemble full-length cDNA of VEE IE. The full length infectious

35   clone is useful, for example, in the production of

8

virulent VEE IE virus, and introducing and testing
attenuating mutations.

In the case of VEE IIIA, the structural protein
genes were removed from a full length clone of VEE

5   IA/B and replaced by the VEE IIIA structural genes.
The IIIA structural gene sequences were prepared by
RT-PCR and include the IIIA 26S promoter and the 3'
nontranslated region (3'NTR) flanking the sequences
for the structural proteins (SEQ ID NO:3). The VEE

10   IIIA 3' NTR was then replaced with the VEE IA/B NTR,
and the modified sequence was cloned back into the. VEE
IA/B full length clone. The result was a clone in
which the nonstructural protein gene sequences were
from VEE IA/B and the structural protein gene

15   sequences from VEE IIIA. The virus produced from this
chimeric clone replicated efficiently in cell culture,
and proved to be completely attenuated in mice. In
addition, it was highly immunogenic and protected the
vaccinated mice against challenge with virulent, wild-

20   type Mucambo virus (VEE IIIA).

Portions of the cDNA sequences described above
are useful as probes to diagnose the presence of virus
in samples, and to define naturally occuring variants
of the virus. These cDNAs also make available

25   polypeptide sequences of WEE antigens, EEE antigens,
VEE IE antigens, and VEE IIIA structural polypeptide
antigens encoded within the respective genomes and
permits the production of polypeptides which are
useful as standards or reagents in diagnostic tests

30   and/or as components of vaccines. Antibodies, both
polyclonal and monoclonal, directed against WEE
epitopes, EEE epitopes, VEE IE epitopes, or VEE IIIA
epitopes contained within these polypeptide sequences
are also useful for diagnostic tests, as therapeutic

35   agents, and for screening of antiviral agents.

Accordingly, with respect to polynucleotides, some aspects of the invention are: a purified WEE polynucleotide; a recombinant WEE polynucleotide; a recombinant polynucleotide comprising a sequence

5   derived from a WEE genome or from WEE cDNA; a recombinant polynucleotide encoding an epitope of WEE; a recombinant vector containing any of the above recombinant polynucleotides, and a host cell transfected with any of these vectors.

10   Other aspects of the invention are: a purified VEE IE polynucleotide; a recombinant VEE IE polynucleotide; a recombinant polynucleotide comprising a sequence derived from a VEE IE genome or from VEE IE cDNA; a recombinant polynucleotide

15   encoding an epitope of VEE IE; a recombinant vector containing any of the above recombinant polynucleotides, and a host cell transformed with any of these vectors.

In a further aspect of the invention is a

20   complete sequence of the VEE subtype IIIA structural protein genes useful for diagnostics and vaccine development. Also provided is a chimeric virus containing the structural sequences of VEE IIIA, which is completely attenuated and provides protection

25   against challenge with VEE IIIA virulent virus for use as a vaccine

Another aspect of the invention is a single-stranded DNA sequence comprising a cDNA clone coding for an infectious WEE, the cDNA clone including at

30   least one attenuating mutation therein, the RNA produced from transcription of the cDNA and the virus particles produced from the RNA in a host cell for use as a vaccine.

In another aspect of the invention there is

35   provided a full length WEE cDNA clone containing a

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defined deletion mutation useful for attenuating the
virus for the identification of suppressor mutations
in the virus. The attenuated virus with the cleavage
deletion and suppressor mutations are useful as a
5   means to generate an attenuated, live WEE virus
vaccine for veterinary and human use.

In a further aspect of the invention is provided
a chimeric virus containing nonstructural protein gene
sequences from WEE and structural protein gene
10 sequences from any alphaviruses including but not
limited to Aura, Barmah Forest, Bebaru, Cabassou,
Chikungunya, eastern equine encephalitis, Everglades,
Fort Morgan, Getah, Highlands J, Kyzylagach, Mayaro,
Middelburg, Mucambo, Ndumu, O'nyong-nyong, Pixuna,
15 Ross River, Sagiyama, Semliki Forest, SAAR87, Sindbis,
Tonate, Una, Venezuelan equine encephalitis, Whataroa,
which could be used as a means for attenuating
virulent alphaviruses, and vaccine production against
other alphaviruses.

20   Taking advantage of the close evolutionary
relationship between WEE and eastern equine
encephalitis virus (EEE), a chimeric virus has been
constructed in which the structural protein genes of
EEE have been inserted into the infectious clone in
25 place of the WEE structural protein genes. The
resulting virus is fully replication competent,
attenuated, and elicits an immune response in mice
which is protective against a lethal challenge with
virulent EEE virus.

30   In addition, depending on the non-WEE sequences
substituted for the WEE structural genes, another
aspect of the invention includes a means for
expressing antigens of other alphaviruses as chimeric
alphaviruses for use as potential vaccines for human
35 and veterinary use.

11

In another aspect of the invention there is
provided a full length infectious VEE IE cDNA clone
containing a cleavage deletion useful in the
identification of suppressor mutations in the virus,

5   the RNA produced from the cDNA and the virus produced
from the RNA. The virus with the cleavage deletion
and suppressor mutations is useful as a means to
generate an attenuated, live VEE IE vaccine virus for
veterinary and human use.

10   In a further aspect of the invention is provided
a chimeric virus containing nonstructural sequences
from VEE IE and structural sequences from other
alphaviruses which could be used as a means of
attenuating virulent alphaviruses.

15   In addition, depending on the non-VEE IE
sequences substituted for the structural sequences of
VEE IE, another aspect of the invention includes a
means to express antigens of other alphaviruses as
chimeric alphaviruses as potential vaccines for human

20   and veterinary use.

In a further aspect of the invention, there is
provided a vaccine protective against WEE, the vaccine
comprising live attenuated WEE virus in an amount
effective to elicit protective antibodies in an animal

25   to WEE and a pharmaceutically acceptable diluent,
carrier, or excipient.

In yet a further aspect of the invention, there
is provided a vaccine protective against VEE IE, the
vaccine comprising live attenuated VEE IE virus in an

30   amount effective to elicit protective antibodies in an
animal to VEE IE and a pharmaceutically acceptable
diluent, carrier, or excipient.

In another aspect of the invention, there is
provided a bivalent vaccine protective against WEE and

35   VEE IE, the vaccine comprising both attenuated WEE and

12

attenuated VEE IE in an amount effective to elicit
protective antibodies in an animal to both WEE and VEE
IE and a pharmaceutically acceptable diluent. In
addition, it is possible that this vaccine will
5   provide short lived protection against other
alphaviruses (Cole and McKinney [1971] *Inf. Immunity*
4:37-43).

     In yet another aspect of the invention, there is
provided an inactivated vaccine produced from the live
10   attenuated virus described above. The attenuated
virus of the present invention whether whole virus or
chimearic virus can be used in producing inactivated
virus vaccines. By using an attenuated virus strain,
there is a much greater margin of safety in the event
15   that the produce is incompletely inactivated.
Starting with an attenuated strain is also much safer
during the manufacturing phase, and allows production
under lower biocontainment levels.

20   **BRIEF DESCRIPTION OF THE DRAWINGS**
     These and other features, aspects, and advantages
of the present invention will become better understood
with reference to the following description and
appended claims, and accompanying drawings where:
25

     Figure 1A, 1B and 1C. Assembly of the full
length cDNA clone of western equine encephalitis
virus. Polymerase chain reaction products
representing the entire genome of WEE virus were
30   prepared with the primer pairs indicated. Each of the
products was cloned into pBluescript KS+. Assembly of
the full length clone, pWE2000 in pBluescript was
carried out as indicated in the figure and described
below. The clones are not drawn to scale. In each of
35   the plasmids, the primers used to generate the PCR

13

products are indicated as are the restriction
endonuclease sites used for the assembly.

Figure 2. Polypeptide profiles of western equine
5   encephalitis viruses. Samples of purified virus were
analyzed by electrophoresis on 10% polyacrylamide gels
and stained with Comassie Brilliant blue. Molecular
weight marker (MW) are shown in the first lane and
molecular weights are indicated in daltons X 10^{-3}. The
10 virus strain is designated above the appropriate lane.
Position of contaminating bovine serum albumin is
indicated by arrow.

Figure 3. Polypeptide profiles of western equine
15 encephalitis virus (WEE), eastern equine encephalitis
(EEE) and chimeric virus, MWE. Samples of purified
virus were analyzed by electrophoresis on 10%
polyacrylamide gels and stained with Comassie
Brilliant blue. Molecular weight markers (MW) are
20 shown and molecular weights are indicated in daltons X
10^{-3}. Position of contaminating bovine serum albumin
is indicated by arrow.

Figure 4. Derivation of virulent VEE IE clone.
25 Constructions of cDNA encoding the entire genome of
cloned strain 68U201 are shown. The relevent cloning
sites and genetic markers are indicated above each
clone. The first full-length clone produced that
replicated *in vitro* after transfection of the RNA
30 transcribed from the T7 promoter, was pIE-1006.
Further modifications to this clone were carried out
in order to obtain pIE-1009 that had the *in vitro* and
in vivo characteristics of the parental, biological
isolate, strain 68U201. With a fully virulent clone,
35 pIE-1009 available, specific attenuating mutations

14

were introduced into pIE-1009 to generate the
attenuated clone, pIE1100. Drawings are not to scale.
Shaded areas represent regions of pIE1006 that were
replaced or mutated to generate new clones.

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     Figure 5. Construction of IAB-IIIA cDNA chimeric
clone pV3A-1000.

DETAILED DESCRIPTION

10   In one embodiment, the present invention relates
to a full length cDNA clone of fully virulent WEE
virus specified in SEQ ID NO:1, and a full length cDNA
clone of fully virulent VEE IE variant specified in
SEQ ID NO:2.

15   WEE, strain CBA/87, isolated from the brains of
an infected horse in Argentina in 1987 (Bianchi *et
al*., [1987] *Am.J. Trop. Med. Hyg.* **49**:322-328) was used
as a parent strain in the instant invention. Any other
strain which consistently kills 100% of 5 week old

20 C57BL6 mice when inoculated subcutaneously can be used
such as B11, for example. The cDNA clone can be
generated by any of a variety of standard methods
known in the art. Preferably, DNA representing the
entire genome can be prepared by polymerase chain

25 reaction using a series of primer pairs based upon the
partial genome sequences previously deposited in
GenBank. The 5' terminal sequence of the virus may be
determined by 5'-RACE basically as described by
Frohman *et al*.([1988] *Proc. Natl. Acad. Sci. U.S.A.*

30 **85**:8998-9002). Assembly of the full length clone can
be in a suitable transcription vector such as, for
example, pBluescript KS+, using convenient restriction
endonuclease sites or the entire genome could be
inserted into any plasmid which contains suitable

35 restriction endonuclease cleavage sites for cloning,

15

an origin of replication so that the plasmid can be
propagated in a bacterial host, and a selectable
marker gene to maintain the plasmid in the bacterial
cell during growth. The DNA sequence preferably has a
5   complementary DNA sequence bonded thereto so that the
double-stranded sequence will serve as an active
template for RNA polymerase. Hence, the transcription
vector preferably comprises a plasmid. When the DNA
sequence comprises a plasmid, it is preferred that a
10   unique restriction site be provided 3' (with respect
to the virion RNA sequence) to ("down-stream" from)
the cDNA clone. This provides a means for linearizing
the DNA sequence to enhance the efficiency of
transcription of genome-length RNA *in vitro*.
15   The complete clone is preferably operatively
associated with a promoter region such that the
promoter causes the cDNA clone to be transcribed in
the presence of an RNA polymerase which binds to the
promoter. The promoter is positioned on the 5' end
20   (with respect to the virion RNA sequence), or upstream
from, the cDNA clone. An excessive number of
nucleotides between the promoter sequence and the cDNA
clone will result in the inoperability of the
construct. Hence, the number of nucleotides between
25   the promoter sequence and the cDNA clone is preferably
not more than eight, more preferably not more than
than 5, still more preferably not more than 3, and
most preferably not more than 1. Exemplary promoters
useful in the present invention include, but are not
30   limited to, T3 promoters, T7 promoters, and SP6
promoters. It is preferable that the 5' end of the *in
vitro* transcript not have any additional nucleotides
preceding the first nucleotide of the viral sequence.
At the 3' end, additional nucleotides can be tolerated
35   in the *in vitro* transcript but are probably lost when

the virus replicates. In most instances, the poly-dA
tract at the 3' end is required for viability of the
virus. Selection of the virulent full length clone
can be achieved by comparing the LD_{50} of the virus
5   encoded by the cloned cDNA with the LD_{50} of the parent
virus used, in the instant example, WEE CBA/87. The
ability to produce virulent virus is important; it
allows the introduction and testing of attenuation
mutations and the attenuated phenotype against a
10   standard: the virulent parent.

Transfection of cells with the RNA transcript
coded by the full length genomic cDNA can be achieved
by any suitable means, such as, for example, by
treating the cells with DEAE dextran, treating the
15   cells with "LIPOFECTIN", and by electroporation.
Togavirus-permissive cells, alphavirus-permissive
cells, and WEE-permissive and VEE IE-permissive cells
are cells which, upon transfection with the viral RNA
transcript, are capable of producing viral particles.
20   Togaviruses have a broad host range. Examples of such
cells include, but are not limited to, Vero cells,
baby hamster kidney cells, chicken embryo fibroblast
cells, Chinese hamster ovary cells (CHO), mouse L
cells, MRC-5 cells, mosquito cells such as C6-36
25   cells, to name a few.

In the case of VEE IE, an isolate of VEE IE,
strain 68U201, was used. Any isolate known to cause
disease in man can be chosen. In addition the ability
of a strain to have an easily identifiable phenotype
30   such as, for example, the ability to form large
plaques in tissue culture on indicator cell
monolayers, is helpful. In the present invention, the
full length clone of VEE IE was obtained using
oligonucleotide primers specific for the VEE IE strain
35   68U201 sequence. Reverse transcription-polymerase

chain reaction (RT-PCR) of strain 68U201 viral RNA was
carried out to generate numerous cDNA fragments that
were subsequently cloned and used to assemble a full-
length cDNA in a plasmid such that the cDNA could be
5   precisely transcribed and viral infectious RNA
produced. It is also possible to produce the entire
genome by polymerase chain reaction by including the
promoter sequence in the 5' end primer thereby
producing infectious RNA directly from the PCR
10   fragment. It is also possible to transcribe the viral
RNA from a plasmid in the cell by transfection with
the appropriate plasmid containing a promoter utilized
by cellular RNA polymerases, i.e. the CMV promoter.
      To determine virulence of the cloned viral
15   genome, mice can be innoculated subcutaneously with
10^4 plaque forming units of the cloned virus. The
clone is considered virulent if all mice die, and not
fully virulent if mice do not all die. The LD_{50} of
parent VEE IE strain 68U201 is to 1-2 pfu, therefore,
20   if inoculation of 10,000-fold did not cause lethal
disease in all mice, it was considered attenuated.
      In another embodiment of the present invnetion is
provided a cDNA sequence of the entire 26S region of
VEE subtype IIIA as well as the structural protein
25   genes specified in SEQ ID NO:3.
      DNA or polynucleotide sequences to which the
invention also relates include sequences of at least
about 6 nucleotides, preferably at least about 8
nucleotides, more preferably at least about 10-12
30   nucleotides, most preferably at least about 15-20
nucleotides corresponding, i.e., homologous to or
complementary to, a region of the WEE, VEE IE, or VEE
IIIA nucleotide sequence. Preferably, the sequence of
the region from which the polynucleotide is derived is
35   homologous to or complementary to a sequence which is

18

unique to the virus. Whether or not a sequence is
unique to the virus can be determined by techniques
known to those of skill in the art. For example, the
sequence can be compared to sequences in databanks,

5   e.g., GenBank. Regions from which typical DNA
sequences may be derived include but are not limited
to, for example, regions encoding specific epitopes,
as well as non-translated regions.

      The derived polynucleotide is not necessarily

10   physically derived from the nucleotide sequence of the
alphaviruses, but may be generated in any manner,
including for example, chemical synthesis or DNA
replication or reverse transcription or transcription,
which are based on the information provided by the

15   sequence of bases in the region(s) from which the
polynucleotide is derived. In addition, combinations
of regions corresponding to that of the designated
sequence may be modified in ways known in the art to
be consistent with an intended use. The sequences of

20   the present invention can be used in diagnostic assays
such as hybridization assays and polymerase chain
reaction assays and for the discovery of other
alphavirus sequences.

      A polypeptide or amino acid sequence derived from

25   the amino acid sequence of alphavirus, refers to a
polypeptide having an amino acid sequence identical to
that of a polypeptide encoded in the sequence, or a
portion thereof wherein the portion consists of at
least 2-5 amino acids, and more preferably at least 8-

30   10 amino acids, and even more preferably at least 11-
15 amino acids, or which is immunologically
identifiable with as a polypeptide encoded in the
sequence.

      A recombinant or derived polypeptide is not

35   necessarily translated from a designated nucleic acid

19

sequence, it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system.

Once a complete viral genomic cDNA is cloned,
5   attenuation of the virus is possible. An attenuating mutation refers to a nucleotide mutation or amino acid coded for in view of such a mutation which results in a decreased probability of causing disease in its host (i.e., a loss of virulence), in accordance with
10   standard terminology in the art. The attenuating mutation may be a substitution mutation or an in-frame deletion mutation.

Novel WEE attenuating mutations disclosed herein which may be used to carry out the present invention
15   include deletion of five amino acids at the furin cleavage site in combination with a substitution of lysine for glutamic acid at codon 182 at E2, or deletion of five amino acids at the furin cleavage site in combination with a subsitution of lysine for
20   glutamic acid at codon 181 at E2. Additionally, certain mutations placed in the non-coding region at the 5' end of the genome have been found to be attenuating, specifically, a C to T change at nucleotide 7, an A to G change at nucleotide 13, a T
25   to A change at nucleotide 25 and deletion of an A at nucleotide 22. These novel attenuating mutations may be inserted together in a cDNA clone encoding WEE virus resulting in an attenuated WEE which is reflected by 100% survival of mice inoculated by
30   subcutaneous and intracranial routes. Such an attenuated live virus is immunogenic and protective against a lethal virus challenge.

Novel attenuating mutations can be discovered in the VEE IE by introducing mutations which are not
35   reparable by the viral RNA replication process. A

20

preferable mutation is the deletion of the four amino
acids of the furin-like cleavage site between the E3
and E2 proteins. Transfection of the mutant viral
genome into cells can result in the suppression of the
5   lethal effect of the deletion mutation due to the
error prone process of alphavirus replication. Once
efficiently replicating viral progeny is generated
they can be detected by plaque assays and analyzed for
the presence of pE2 protein which indicates that the
10   virus contains the deletion mutation. Attenuated but
yet immunogenic virus with a cleavage deletion
mutation and suppressor mutation(s) could be tested
for its ability to protect animals from challenge with
virulent VEE IE.
15   Attenuating mutations may be introduced into
cDNAs encoding live WEE or VEE IE by any suitable
means, such as site-directed mutagenesis (Please see
e.g., Maniatis, Fritsch and Sambrook, Molecular
Cloning: A Laboratory Manual (1982) or DNA Cloning,
20   Volumes I and II (D. N. Glover ed. 1985) or Current
Protocols in Molecular Biology, Ausubel, F. M et al.
(Eds.) John Wiley & Sons, Inc., for general cloning
methods.).
        In another embodiment of the present invention is
25   provided a chimeric virus containing nonstructural
sequences from one alphavirus and structural sequences
from other alphaviruses which could be used as a means
of attenuating virulent alphaviruses. By "Structural
sequences" as used herein is meant sequences encoding
30   proteins which are required for encapsidation (e.g.,
packaging) of the viral genome, and include the capsid
protein, E1 glycoprotein, and E2 glycoprotein. By
"nonstructural sequences" is meant nonstructural
protein sequences, or sequences which encode viral RNA

polymerase(s) proteins. Viruses from which structural
sequences can be used in the chimeric virus using WEE
"nonstructural genes" as the backbone clone can
include for example, all strains of WEE, EEE, and

5   Sindbis, Aura, Barmah Forest, Bebaru, Bijou Bridge,
Cabassou, Chikungunya, Everglades, Fort Morgan, Getah,
Highlands J, Kyzylagach, Mayaro, Middelburg, Mucambo,
Ndumu, O'nyong-nyong, Pixuna, Ross River, Sagiyama,
Semliki Forest, SAAR87, Tonate, Una, Venezuelan Equine

10   Encephalitis, Whataroa, to name a few. Acceptable
structural protein genes would include a nucleocapsid
protein capable of both packaging the chimeric viral
genome and which can interact with the glycoproteins
to initiate particle assembly. Chimearic virus is

15   constructed by excision of the structural protein
genes of the backbone virus and replacement with the
desired structural protein genes from another virus.
This can be accomplished in one of several ways. For
example, site-directed mutagenesis can be used to

20   excise the structural protein genes and leave a
restriction endonuclease digestion site at the point
of deletion. The structural protein genes of another
alphavirus would then be cloned into that restriction
site. Any virus obtained after transfection of cells

25   with RNA transcribed from that clone would by
definition be a chimeric virus.

          In the case where the first and second viruses
are closely related, another method can be used
wherein cloned structural cDNA sequences of a second

30   alphavirus can be digested at restriction enzyme sites
which both viruses have in common. The cDNA fragments
of the second virus can then be cloned into the
homologous sites in the first virus structural protein
locus such that the resulting structural protein genes

35   of the chimeric are a composite of both. Other

22

methods for producing a chimearic virus are known to people in the art (Kuhn *et al.* [1996] *J. Virology* **70**:7900-7909).

5   In another embodiment, the attenuated viruses of the present of invention can be used to prepare replicon expression systems. A replicon expression system consists of three components. The first is a replicon which is equivalent to a full length infectious clone from which all of the viral

10   structural proteins have been deleted. A multiple cloning site can be cloned into the site previously occupied by the structural protein genes. Virtually any heterologous gene may be cloned into this cloning site. Transcription of RNA from the replicon yields

15   an RNA capable of initiating infection of the cell identically to that seen with the full-length infectious virus clone. However, in lieu of the viral structural proteins, the heterologous antigen is expressed. This system does not yield any progeny

20   virus particles because there are no viral structural proteins available to package the RNA into particles.

       Particles which appear structurally identical to virus particles can be produced by supplying structural proteins for packaging of the replicon RNA

25   in *trans*. This is typically done with two helpers. One helper consists of a full length infectious clone from which the nonstructural protein genes and the glycoprotein genes are deleted. The helper retains only the terminal nucleotide sequences, the promoter

30   for subgenomic mRNA transcription and the sequence for the viral nucleocapsid protein. The second helper is identical to the first except that the nucleocapsid gene is deleted and only the glycoprotein genes are retained. The helper RNA's are transcribed *in vitro*

35   and co-transfected with replicon RNA. Because the

23

replicon RNA retains the sequences for packaging by the nucleocapsid protein, and because the helpers lack these sequences, only the replicon RNA is packaged by the viral structural proteins and released from the

5   cell. The particles can then be inoculated into animals similar to parent virus. The replicon particles will initiate only a single round of replication because the helpers are absent, they produce no progeny virus particles, and express only

10   the viral nonstructural proteins and the product of the heterologous gene cloned in place of the structural proteins. The heterologous gene product is then detected by the host immune system and appropriate immune response is then mounted.

15   The WEE and VEE IE replicons can be used to express heterologous genes of interest as well as a means for expressing antigens or immunogenic proteins and peptides of interest, *in vitro* or *in vivo*. The immunogenic protein or peptide, or "immunogen" may be

20   any immunogen suitable for inducing an immune response protective against a pathogen from which the immunogen is derived, including but not limited to microbial, bacterial, protozoal, parasitic, and viral pathogens. For example, the immunogen can be the expression

25   product of any heterologous gene of interest, including influenza hemagglutinin, lassa fever nucleocapsid and glycoproteins, portions of bacterial toxin genes, HIV glycoprotein, Ebola glycoprotein, to name a few.

30   In yet another embodiment, the present invention provides inactivated virus vaccines produced from live attenuated virus preparations, either as virus with attenuating mutations as has been described for WEE and VEE IE or chimeric viruses described above for EEE

35   and VEE IIIA. The inactivation of live virus is well

24

known in the art and can be performed, for example, by the use of formalin. Inactivated attenuated virus vaccine has a greater safety margin both as a final vaccine in case of incomplete inactivation, and during
5 the manufacturing process allowing production under lower biocontainment levels.

Subjects which may be administered the live attenuated or inactivated attenuated viruses and vaccine formulations disclosed herein include both
10 humans and animals (e.g. horse, donkey, pigs, mice, hamster, monkey, birds).

Vaccine formulations of the present invention comprise an immunogenic amount of a live attenuated virus, or a combination of live attenuated viruses as
15 a multivalent vaccine, as disclosed herein in combination with a pharmaceutically acceptable carrier. An "immunogenic amount" is an amount of the attenuated virus sufficient to evoke an immune response, particularly an immune response to the
20 protein or peptide encoded by the heterologous RNA carried by the virus, in the subject to which the virus is administered. An amount of from about 10^1 to 10^5 plaque forming units of the live virus per dose is suitable, depending upon the age and species of the
25 subject being treated. Exemplary pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution.

Administration of the live attenuated viruses
30 disclosed herein may be carried out by any suitable means, including both parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), by in ovo injection in birds, and by topical application of the virus (typically carried in
35 the pharmaceutical formulation) to an airway surface.

Topical application of the virus to an airway surface
can be carried out by intranasal administration (e.g.
by use of dropper, swab, or inhaler which deposits a
pharmaceutical formulation intranasally). Topical

5   application of the virus to an airway surface can also
be carried out by inhalation administration, such as
by creating respirable particles of a pharmaceutical
formulation (including both solid particles and liquid
particles) containing the virus as an aerosol

10   suspension, and then causing the subject to inhale the
respirable particles. Methods and apparatus for
administering respirable particles of pharmaceutical
formulations are well known, and any conventional
technique can be employed.

15   In another embodiment, the present invention
relates to antibodies specific for the above-described
virus. For instance, an antibody can be raised
against any of the viral proteins or against a portion
thereof. Persons with ordinary skill in the art using

20   standard methodology can raise monoclonal and
polyclonal antibodies to a polypeptide of the present
invention. Material and methods for producing
antibodies are well known in the art (see for example
Goding, in, Monoclonal Antibodies: Principles and

25   Practice, Chapter 4, 1986). The antibodies can be
used to monitor the presence or activity of
alphaviruses and potentially as a therapeutic agent.

          In a further embodiment, the present invention
relates to a method of detecting the presence of WEE,

30   EEE, VEE IIIA or VEE IE viral infection or antibodies
against these viruses, if present, in a sample. Using
standard methodology well known in the art, a
diagnostic assay can be constructed by coating on a
surface (i.e. a solid support) for example, a

microtitration plate or a membrane (e.g.
nitrocellulose membrane), all or a unique portion of
WEE, EEE, VEE IIIA or VEE IE virus described above,
and contacting it with the serum of a person suspected

5   of having a viral infection. The presence of a
resulting complex formed between the virus and
antibodies specific therefor in the serum can be
detected by any of the known methods common in the
art, such as colorimetry or microscopy. This method

10   of detection can be used, for example, for the
diagnosis of WEE, EEE, VEE IIIA and VEE IE viral
infections.

In yet another embodiment, the present invention
relates to a method of detecting the presence of WEE,

15   EEE, VEE IIIA or VEE IE viruses in a sample. Using
standard methodology well known in the art, a
diagnostic assay can be constructed by coating on a
surface (i.e. a solid support) for example, a
microtitration plate or a membrane (e.g.

20   nitrocellulose membrane), antibodies specific for WEE
and/or VEE IE, and contacting it with serum or tissue
sample of a person suspected of having a WEE or VEE IE
viral infection. The presence of a resulting complex
formed between virus in the serum and antibodies

25   specific therefor can be detected by any of the known
methods common in the art, such as fluorescent
antibody spectroscopy or colorimetry. This method of
detection can be used, for example, for the diagnosis
of WEE, EEE, VEE IIIA and VEE IE viral infection.

30   In another embodiment, the present invention
relates to a diagnostic kit which contains WEE, EEE,
VEE IE, or VEE IIIA virus and ancillary reagents that
are well known in the art and that are suitable for
use in detecting the presence of antibodies to WEE and

35   VEE IE in serum or a tissue sample. Tissue samples

27

contemplated can be obtained from birds, monkey, human, or other mammals.

In yet a further embodiment, the present invention relates to DNA or nucleotide sequences for
5   use in detecting the presence or absence of WEE, EEE, VEE IE or VEE IIIA virus using the reverse transcription-polymerase chain reaction (RT-PCR). The DNA sequence of the present invention can be used to design primers which specifically bind to the viral
10   RNA for the purpose of detecting the presence, absence, or quantitating the amount of virus. The primers can be any length ranging from 7-40 nucleotides, preferably 10-15 nucleotides, most preferably 18-25 nucleotides. Reagents and controls
15   necessary for PCR reactions are well known in the art. The amplified products can then be analyzed for the presence or absence of viral sequences, for example by gel fractionation, with or without hyridization, by radiochemistry, and immunochemical techniques.
20   In yet another embodiment, the present invention relates to a diagnostic kit which contains PCR primers specific for WEE, EEE, VEE IE or VEE IIIA and ancillary reagents that are well known in the art and that are suitable for use in detecting the presence or
25   absence of WEE, EEE, VEE IE or VEE IIIA in a sample using PCR. Samples contemplated can be obtained from birds, human, or other mammals.

In another embodiment, the present invention relates to a method of reducing WEE, EEE, VEE IE, or
30   VEE IIIA viral infection symptoms in a patient by administering to said patient an effective amount of anti WEE, anti EEE, anti VEE IE, or anti VEE IIIA antibodies, or protective serum from an immunized animal. When providing a patient with antibodies, the
35   dosage of administered agent will vary depending upon

such factors as the patient's age, weight, height,
sex, general medical condition, previous medical
history, etc. In general, it is desirable to provide
the recipient with a dosage of the above compounds

5   which is in the range of from about 1pg/kg to 10 mg/kg
body weight of patient, although a lower or higher
dosage may be administered.

In another embodiment, the present invention
relates to a method for overcoming vaccine

10 interference in alphavirus-immune individuals.
Alphavirus interference has been documented in animals
and people since the 1960's. This phenomenon occurs
when a live-attenuated vaccine is administered to
animals or people with existing immunity to

15 heterologous alphaviruses. Pre-existing immunity may
be acquired by vaccination or infection. This
presents a significant limitation to the usefulness of
the current live-attenuated alphavirus vaccines,
especially since the cross-reactive immunity does not

20 protect adequately against challenge with virulent
heterologous alphaviruses. Formalin-inactivated
vaccines are not an acceptable alternative as they
have significant limitations with regard to the
quality and duration of protective immunity and

25 require multiple inoculations and periodic boosters.
The attenuated WEE, EEE, VEE IIIA and VEE IE virus
vaccines of the present invention contain mutations in
the viral glycoprotein sequences that may alter the
sequence, conformation, and/or accessibility of cross-

30 reactive epitopes. Alterations in epitopes that
prevent binding by cross-reactive antibodies may also
bypass interference in alphavirus-immune individuals.
Eliminating the problem of interference would permit
the WEE and VEE IE attenuated virus vaccines to be

35 used in alphavirus-immune animals or people to induce

protective immunity to western equine encephalitis
virus and/or to Venezuelan equine encephalitis virus
variant IE. Long-lasting protective immunity to both
parenteral and aerosol challenge would be expected
5   after vaccination with the live-attenuated vaccines of
the present invention, and provide an additional
advantage over the use of inactivated vaccines which
induce short-lived responses that do not protect
against mucosal challenge.

10   Having now described the invention, the following
examples are provided to illustrate the present invention,
and should not be construed as limiting thereof. In light
of the present disclosure, numerous embodiments within the
scope of the claims will be apparent to those of ordinary
15   skill in the art.

Attenuated WEE

The following materials and methods were used in
20   the examples that follow.

Viruses and cells. Western equine encephalitis
virus, strain CBA/87 (Bianchi et al. 1988) and eastern
equine encephalitis virus, strain Fla91-4679 (Mitchell
et al.[1992] Science 257:526-7) were grown in BHK
25   cells in EMEM containing 10% fetal bovine serum, 100
U/ml penicillin G and 100 µg/ml streptomycin. Primary
chicken embryo fibroblasts were grown in EMEM
containing 5% fetal bovine serum. Baby hamster kidney
cells and Vero cells were grown in EMEM containing 10%
30   fetal bovine serum.

cDNA cloning. Genomic RNA was prepared from
purified virus by phenol:chloroform extraction and
ethanol precipitation. Initially, cDNA was prepared
by the method of Gubler and Hoffman ([1983] Proc.

Natl. Acad. Sci. U.S.A. **85**:5997-6001). Subsequently,
DNA representing the entire genome was prepared by
polymerase chain reaction using a series of primer
pairs (Table 1, Figure 1) based upon the partial

5   genome sequences previously deposited in GenBank.
Each PCR product was cloned into pCRII (Invitrogen).
The 5' terminal sequence of CBA/87 virus was
determined by 5'-RACE basically as described by
Frohman *et al.* ([1988] *Proc. Natl. Acad. Sci. U.S.A.*

10   **85**:8998-9002). The oligonucleotide, CBA/87 5',
consisted of an *Sst*II site, the promoter for
bacteriophage T7 RNA polymerase followed by one G and
14 nucleotides of the 5' terminus of CBA/87 terminal
sequence was paired with ns1962 and used to amplify

15   the terminal 1.9 kb of the WEE genome. Clone pWE2000
representing the entire genome was assembled in
pBluescript KS+ through the use of convenient
restriction endonuclease sites.

20   Table 1. Oligonucleotide Primers for Preparation of
     WEE PCR Products
     CBA/87 -T7-Sst2
            GTCACCGCGGTAATACGACTCACTATAGATAGGGCATGGTATAGAG
                    (SEQ ID NO:4)
25   NS1962   TCACCTTATTCTGGAACACATCAG (SEQ ID NO:5)
     WEE-7   TCGGAGGAAGGCTGATGAAAC (SEQ ID NO:6)
     WEE-10   TCGGATCCGATGAGAAAATATACGCTCCC (SEQ ID NO:7)
     WEE-17   GACTGGATCCGCAAACCAGTCCTGTTCTCAGG
                    (SEQ ID NO:8)
30   WEE-18   GCATGGATCCAGCATGATCGGAAATGTCTTGTC
                    (SEQ ID NO: 9)
     WEE-5   TCGGATCCACCGCCAAAATGTTTCCATAC (SEQ ID NO:10)
     WEE-3   TCGGGATCCCCGGAACATTTGGC (SEQ ID NO:11)
     WEE-2   CTGCTTTTCATGCTGCATGCC (SEQ ID NO:12)
35   WEE-Not   CGATGCGGCCGCTTTTTTTTTTTTTTTTTTGAAATTTTAAAAAC
                    (SEQ ID NO:13)
     WEE-CL2   CAGCGTGAAGTCATCGGTAATGCTGCGTGATGGACATTTCAAG
                    (SEQ ID NO:14)
     WEE-CL1   CAGCGTGAAGTCATCGGTAATGCTTGATGGACATTTCAAG
40   (SEQ ID NO:15)

31

For ease of subsequent site-directed mutagenesis
of the structural protein genes, two cassettes
representing the 5' terminal 7.6 kb, plasmid pWE5'-18,
and 3' 4.2 kb, plasmid pWE3'-17, of the genome were
5   prepared. Full length clones were assembled by
digestion of the pWE5'-18 with *BlnI* and *NotI* and
insertion of a 4.1 kb *BlnI-NotI* fragment prepared from
the plasmid pWE3'-17 or its mutagenized derivatives.
A cassette containing the structural genes of
10   eastern equine encephalitis virus strain Fla91-4679
was prepared by RT-PCR. The cassette was digested with
BlnI and *NotI* and the 4.0 kb fragment was ligated to
pWE5'-18 which had been similarly digested. The
resulting plasmid was designated pMWE-7.
15   <u>Mutagenesis of the furin cleavage site</u>. Two
oligonucleotides, WEE-CL2 and WEE-CL which bracket the
presumed furin cleavage site, RRPKR, between the E3
and E2 glycporoteins were used to delete the 5 and 4
codons, respectively. Plasmid pWE3'-17 was used as
20   template for mutagenesis. WEECL2 and WEECL were
paired with primer WEE-5 to generate PCR products of
1.4 kb. The PCR products were purified and paired
with WEE-3 for 10 cycles of PCR utilizing WE3'-17 as
template. Additional WEE-5 primer was added to 500 nM
25   and PCR was carried out for an additional 20 cycles.
The 2.3 kb products were purified, digested with
BstEII and *NcoI* and ligated into plasmid pWE3'-17
which had been digested with *BstEII* and *NcoI*. Clones
containing the mutation were identified by loss of the
30   *NgoM1* site in the sequences encoding the furin
cleavage site. The sequences of the mutations were
confirmed by sequencing and the mutagenized pWE3'-17
cassettes were ligated to pWE5'-18 as described above
to yield full length clones pWE2200 and pWE2100,
35   respectively.

Identification of secondary mutations in virus
derived from pWE2100 and pWE2200. Virus released from
cells electroporated with RNA transcribed from pWE2200
were plaque-purified and grown into stock

5   preparations. Supernatant from cells transfected with
RNA transcribed from pWE2100 was diluted 10-fold and
inoculated onto BHK cells. The supernatant was
collected 48 hours later and the RNA was extracted
directly from the culture fluid. For each mutant

10 virus and the parent CBA/87 virus, viral RNA was
extracted with Trizol LS (Life Technologies, Inc.,
Gaithersburg, MD) and the glycoprotein genes of each
were amplified by reverse transcription-polymerase
chain reaction amplification. The PCR products were

15 purified (PCR Prep, Promega Inc., Madison, WI) and
sequenced on an ABI 373 sequencer using fluorescent-
tagged terminators.

Transcription and transfection. Purified plasmid
was digested with NotI, phenol extracted and ethanol

20 precipitated. Typically 0.5-1 ug of linearized DNA
was transcribed in vitro by T7 RNA polymerase
(Ribomax, Promega, Madison, WI) in the presence of 3
mM m7GpppGp (Pharmacia, Piscataway, NJ).
Electroporation of BHK or CEF cells with 0.4 ug of RNA

25 was done as described (Liljestrom et al. [1991]
Bio/Technology 9:1356-1361). The cells were then
seeded into T-75 flasks with 20 ml of medium and
observed for cytopathology at 24 and 36 hours after
electroporation. Virus was harvested when the cells

30 displayed significant cytopathology and approximately
50% were detached from the plastic. Virus titers were
determined by plaque assay on Vero and BHK cells.

33

EXAMPLE 1

Preparation of an infectious clone of WEE strain CBA/87.

One goal of this study was to prepare a full
5   length cDNA clone of fully virulent WEE virus such
that mutations leading to an attenuated phenotype
could be identified. WEE, strain CBA/87, isolated
from the brain of an infected horse in Argentina in
1987 (Bianchi *et al.* [1988] *Am. J. Trop. Med. Hyg.*
10 **49**:322-328), was chosen as the parent virus as it
consistently kills 100% of 5 week old C57BL6 mice when
inoculated subcutaneously, allowing development of a
convenient animal model to assess the relative effects
on virulence of the attenuating mutations.   Portions
15 of the sequence of several strains of WEE virus had
been determined previously and were used as a basis
for primers to prepare amplification products
representing the entire genome of the CBA/87 which
were cloned in pCRII. Full length clones were
20 assembled in pBluescript KS+ using convenient
restriction sites as shown in Figure 1. The first
infectious clone pWE1000, contained the 5' terminal 20
nucleotides from eastern equine encephalitis virus.
This clone produced viable virus which was highly
25 attenuated exhibiting an subcutaneous LD_{50} in mice of
approximately 1.2 X 10^6 PFU compared to the CBA/87
parent virus where LD_{50} was approximately 22.

A second clone with an authentic WEE 5'-terminus,
pWE2000, was used for all subsequent experiments.
30 Transfection of CEF or BHK cells with RNA transcribed
from pWE2000 resulted in complete destruction of the
monolayers within 36 hours and titers >10^8 PFU/ml were
obtained by infection of either cell type with the
resulting virus. Subcutaneous inoculation of 5 week

34

old C57/B16 mice with WE2000 results in death within 9
days and the LD_{50} of 75 PFU is only slightly higher
than the LD_{50} of 22 PFU of CBA/87 parent virus, Table
2. It should be noted that animals surviving the

5   lower doses of virus challenge gave no serological
evidence of infection. This level of virulence for
WE2000 virus was considered sufficient to allow for
further characterization of the mutations necessary
for attenuation of the virus.

10
Table 2. C57 Black/6 Mice Inoculated Subcutaneously
with CBA/87 Parental virus and WE2000 Recombinant
Virus

Virus Strain Dose	Mortality	Mean Day to Death (Days)	Prechallenge ELISA	Challenge (S/T)
CBA/87				
$3X10^5$	10/10	8.4	–	–
$3X10^4$	10/10	8.9	–	–
$3X10^3$	10/10	9.2	–	–
$3X10^2$	7/10	9.9	<100	0/3
$3X10^1$	3/10	10	<100	0/7
3	0/10	––	<100	0/10
WE2000				
$1X10^7$	10/10	8.7	–	–
$1X10^6$	10/10	9.0	–	–
$1X10^5$	10/10	9.2	–	–
$1X10^4$	10/10	9.3	–	–
$1X10^3$	9/10	10.0	<100	0/1
$1X10^2$	3/10	10.0	<100	0/7
$1X10^1$	0/10	–	<100	0/10
1	0/10	–	<100	0/10

15

EXAMPLE 2

Preparation of cleavage mutants of CBA/87.

Davis *et al.*([1995] *Virology* **212**:102-110) have
demonstrated that deletion of the furin cleavage site

20   between E3 and E2 glycoproteins of VEE virus is a
lethal mutation. However, prolonged incubation of
cells which had been transfected with RNA derived from

35

full length clones with the deletion, resulted in the
eventual appearance of virus which was replication-
competent and attenuated in mice (Davis *et al.*, 1995,
supra). Based upon a comparison of the predicted
5   structural protein sequences of WEE and VEE, the
probable cleavage site of CBA/87 virus is RRPKR. The
presence of the extra arginine when compared to the
conscensus (RX(R/K)R) alphavirus cleavage site
indicated that the cleavage site of WEE virus might be
10   more complex than that observed with VEE virus.   We
therefore, prepared two different deletion mutations
in the E3-E2 cleavage site of the pWE2000 clone,
pWE2100 which lacks five amino acids, RRPKR, and
pWE2200 which lacks four amino acids, RPKR.
15   Development of CPE in cells after electroporation
of RNA transcribed from pWE2100 and pWE2200 was
delayed for 48 to 72 hours compared with pWE2000. In
two instances, pWE2100 did not induce significant CPE
in the transfected cells despite the fact that
20   approximately 10^6 PFU/ml were released into the medium
of transfected cells. When assayed by plaque
formation on Vero cells, both supernatants yielded
extremely small plaques after 72 hours which never
increased beyond 2 mm in diameter. In contrast,
25   pWE2000 virus yields large plaques after 48 hours
which enlarge to approximately 1 cm after 5 days under
a 0.5% agarose overlay. The small plaque phenotype of
the mutant viruses is stable after 3 passages in Vero
or BHK cells, which is the limit to the passage of the
30   virus used in these experiments.
Analysis of the structural proteins of the WE2100
and WE2200 viruses by SDS-PAGE (Figure 2) shows that
in each instance, the deletions at the cleavage site
result in a virus which lacks E2 protein and contains
35   a larger protein, presumably pE2, indicating that

36

deletion of the presumed cleavage site eliminated
cleavage at this site.

The lack of rapid cytopathology after
transfection of BHK cells with transcripts of pWE2200
5   and pWE2100 suggested that the mutants were non-viable
and that the infectious virus subsequently detected by
plaque assay was due to secondary mutations arising
during the replication of the RNA as reported
previously for deletion mutants of VEE (Davis et al.,
10 1995, supra).

Three plaque isolates from WE2200 virus were
chosen for further characterization. All isolates
grew to high titer and exhibited a small plaque
phenotype. The isolates were sequenced over the
15 entire glycoprotein reading frame. As shown in Table
3, isolates 2215 and 2220 have a mutation of Glu to
Lys at position 181 of the E2 glycoprotein. Strain
WE2219 carries a single Glu to Lys at position 182 of
the E2 protein. Strain WE2215 also has a conservative
20 Val to Ala change at position 211 of the E2
glycoprotein. WE2220 has a Glu to Gly change at
position 2 of the E1 glycoprotein and a Phe to Ser
change at position 382 of the E1 glycoprotein.

25 Table 3. Genotypes of Recombinant WEE Virus Strains

Virus[1]	Cleavage Site	E2[2]	E1[2]
30 CBA/87	RRPKR	P(102),E(181),E(182)	E(2),F(257),P(382)
vWE2100	------	K(182)	
pWE2102	------	K(182)	
35			
vWE2215	R-----	K(181), A(211)	
vWE2219	R-----	K(182)	
vWE2220	R-----	K(181)	G(2), S(382)

37

1. p indicates viruses prepared by mutagenesis of infectious
clones.
2. Amino acid at position indicated in parenthesis.

5   In order to determine a consensus of the
mutations appearing in virus produced from the pWE2100
RNA, the sequences of the glycoprotein genes were
determined directly from cDNA prepared by RT-PCR of
the genomic RNA extracted from virus released from BHK
10   cells infected with virus released from the
transfected cells. Sequence analysis of glycoprotein
genes of WE2100 virus from the transfection
supernatant revealed only two mutations. As seen
previously in WE2219, WE2100 also had a Glu to Lys
15   change at position 182 of the E2 glycoprotein (Table
3).

In order to determine which of the mutations
identified in virus released from cells transfected
with RNA transcripts from pWE2200 and pWE2100 served
20   as the suppressor of the lethal effect of the cleavage
deletion mutation, the mutations were individually
placed into the pWE2200 and pWE2100 clones by site-
directed mutagenesis. As shown in Table 4, three
subclones of WE2200 were produced, and based upon the
25   ability to induce CPE in BHK cells, it was
demonstrated that the Glu to Lys change at position
181 of the E2 glycoprotein was necessary and
sufficient to restore the ability of the WE2200 clone
to encode replication competent virus. Similarly,
30   placement of the Glu to Lys change at position 182 of
the E2 glycoprotein was also sufficient to restore the
ability of the WE2100 clone to encode replication
competent virus. When the Glu to Lys changes at E2
position 181 or 182 were inserted into the parental
35   infectious clone pWE2000, the resulting virus

38

exhibited a small plaque phenotype on Vero cells as
noted for each of the cleavage deletion mutants.

5   Table 4. Effect of site-directed mutagenesis on
     restoration of cytopathogenicity of pWE2200 and
     pWE2100

Strain	Cleavage site	E2	E1	Viability
pWE2000	RRPKR			Yes
pWE2200	R----			No
pWE2221	R----		G(2)	No
pWE2222	R----	K(181)	G(2)	Yes
pWE2223	R----	K(181)		Yes
pWE2100	-----			No
pWE2102	-----	K(182)		Yes

20

        Although the cleavage deletion mutations in
pWE2200 and pWE2100 differed by a single amino acid,
the results indicate that the mutations at E2 residues
181 and 182 are both capable of restoring viability of
25   the virus and appear to be equivalent.

                        EXAMPLE 3
              Attenuation of WE2000 virus.
        C57/BL6 mice are uniformly susceptible to lethal
30   challenge by western equine encephalitis virus until
     approximately 9 weeks of age. Subcutaneous
     inoculation of five- or eight-week-old C57/BL6 female
     mice with CBA/87 or WE2000 viruses routinely results
     in lethal encephalitis after 8-9 days (Table 2). As
35   noted previously, the WE2000 is slightly less virulent
     than the CBA/87 parent. The virulence of the progeny
     virus derived from pWE2100 and pWE2200 infectious
     clones were determined by subcutaneous inoculation of
     C57BL6 mice. In each instance, the viruses were

39

significantly attenuated compared to virus produced from parent WE2000 clone. However, infection of mice with increasing doses of WE2215, WE2219 or WE2220, resulted in sporadic deaths with slightly extended
5 periods prior to death compared to the parental virus (Table 5). These results indicated that deletion of only four amino acids from the cleavage site was inadequately attenuating, unlike the results obtained with VEE virus by Davis *et al.* (1995) and the viruses
10 derived from WE2200 were not characterized further.

Table 5. C57BL6   Mice Inoculated Subcutaneously with WE2200 Cleavage Deletion Mutants

Virus Strain Dose	Mortaliy	Mean day to Death	Prechallenge ELISA Neut	Challenge	Post Challenge ELISA Neut
CBA/87					
10^3	10/10	9.1			
10^5	10/10	7.7			
WE2000					
10^3	10/10	9.2			
10^5	10/10	9.1			
WE2215					
10^3	1/10	12	504 (3/9) <20(9/9) 100 (6/9)	8/9	8300 761
10^5	1/10	14	253 (7/9) <20(9/9) 100 (2/2)	9/9	12800 403
WE2219					
10^3	0/10	–	283(2/8) <20(10/10) 100 (6/8)	6/10	14368 2281
10^5	2/10	12	200 (4/8) <20(8/8) 100 (4/8)	8/8	11738 1522
WE2220					
10^3	1/10	11	606 (5/9) 20(3/9) 100 (4/9) <20 (6/9)	5/9	14703 1280
10^5	2/10	14	800 (7/8) 20 (2/8) 100 (1/8) <20 (6/8)	8/8	9051 761

15

When C57BL6 mice were inoculated subcutaneously
with the uncloned WE2100 progeny virus, there were no
deaths at any dilution, even at doses of 10^7 PFU per

5   mouse (Table 6). However, some of those mice
inoculated subcutaneously with 10^5 PFU or less
remained susceptible to a lethal challenge with the
virulent CBA/87 virus.

10   Table 6. C57 Black/6 Mice Inoculated Subcutaneously
with WEE Virus Strain WE2100

Virus strain Dose	Mortality[1]	(%)	Mean Day to Death	Challenge[2]	(%)
WE2100					
10^3	0/10	(0)	–	4/10	(40)
10^4	0/10	(0)	–	7/10	(70)
10^5	0/10	(0)	–	8/10	(80)
10^6	0/10	(0)	–	10/10	(100)
10^7	0/10	(0)	–	10/10	(100)

1. Expressed as animals dying/animals tested
15   2. Expressed as animals surviving/animals tested

WE2102 virus was demonstrated to be highly
attenuated and killed only two of twenty mice when
20   inoculated subcutaneously with the highest dosage of
virus (10^7 PFU). All mice were challenged 3 weeks
later with 10^5 PFU of CBA/87 virus. Mice previously
immunized with 10^5 PFU or more of WE2102 survived with
no noticeable symptoms. Thus, an effective immunizing
25   dose of WE2102 is at least 100 fold less than that
required to kill C57BL6 mice. These results further
indicate that the Glu to Lys change at position 182 of
the E2 glycoprotein is responsible for restoring
viability to viruses containing a deletion of the
30   furin cleavage site in the WEE glycoproteins and that
WE2102 virus is an effective attenuated vaccine virus.

Table 7.   Subcutaneous Inoculation of C57BL/6 Mice
with recombinant WEE virus strain 2102 confers
protection against a lethal challenge

Virus Strain Dose	Mortality[1] (%)	Mean Day to Death	Challenge[2] (%)
WE2100			
10^3	0/10 (0)		3/10 (30)
10^5	1/10 (10)	16	9/9 (100)
10^7	0/10 (0)		10/10 (100)
WE2102			
10^3	0/20 (0)	–	9/20 (45)
10^5	0/20 (0)	–	18/20 (90)
10^7	2/20 (0)	14	18/18 (100)
WE2000			
10^5	10/10 (100)	9.2	

1.   Expressed as animals dying/animals tested
2.   Expressed as animals surviving/animals tested

Attenuated VEE IE

EXAMPLE 4

Sequence Analysis. Genomic RNA from VEE IE Strain
68U201 was isolated and reverse transcription of the
genomic RNA followed by polymerase chain reaction (RT-PCR)
was used to generate cDNA of the virus. Initial sequencing
of the strain 68U201 genome employed oligonucleotide
primers based on existing VEE IE sequence and VEE IA/B
sequence. After a partial sequence of strain 68U201 was
determined, oligonucleotides specific to the strain 68U201
sequence were used to obtain a complete sequence of the
strain 68U201 viral genome. The exact 5' end of the
genome was determined by PCR/RACE technique (Frohman et
al. [1988] Proc. Natl. Acad. Sci. U.S.A. 85:8998-9002).
The entire strain 68U201 viral genomes consists of 11,464

42

nucleotides, excluding the poly-A sequence (Oberste *et al.* [1996] *Virology* **219**: 314-320).

EXAMPLE 5

5   ## Construction of Full-length, Live Clones

Using oligonucleotides specific to the VEE IE strain 68U201 sequence, RT-PCR of strain 68U201 viral RNA was carried out to generate numerous cDNA fragments that were subsequently cloned. These clones

10   were used to assemble a full-length cDNA of strain 68U201 in a plasmid situated such that the cDNA could be precisely transcribed in an *in vitro* transcription reaction employing T7 polymerase. The first nucleotide downstream of the T7 promoter is a G

15   followed by a cDNA encoding the entire strain 68U201 genome, including poly-A sequence. For the purposes of run off *in vitro* transcription, a unique endonuclease restriction site (*NotI*) follows the poly-A sequence (Figure 4). DNA sequences encoding the T7

20   promoter and the strain 68U201 genome were cloned into a suitable plasmid for propagation and selection in *E. coli*. Oligonucleotides relevant to construction of full-length infectious clones are shown in Table 8.

25   Table 8

```
     0077    CTAAGAGGGGCCCCTATATC (SEQ ID NO:16)
     0111    GCGGAATTCTAATACGACTCACTATAGATGGGCGGCGCATGAGAG
              (SEQ ID NO:17)
30   0113    TGACCGCGGGACCTCTGTCCAC (SEQ ID NO:18)
     0126    AAGTGCATCGATTCAGCG (SEQ ID NO:19)
     0136    CTGAAATGTCCAGGATCCACGGAGGAGCTG (SEQ ID NO:20)
     0137    CAGCTCCTCCGTGGATCCTGGACATTTCAG (SEQ ID NO:21)
     0140    GACTGCGGCCGCTTTTTTTTTTTTTGAAATATTAAAAACAAAATCC
35            (SEQ ID NO:22)
     0220    CGAGAATCGATGCACTTCAGCC (SEQ ID NO:23)
```

Descriptions of oligonucleotides are as follows: 0077 introduces an *ApaI* endonuclease restriction site along with a serine to proline mutation within the coding region of nsP4. 0111 encodes an EcoRI

5   endonuclease restriction site followed by the T7 promoter, a single G, and the first 18 nucleotides of the VEE IE genome. 0126 introduces a *ClaI* endonuclease restriction site within the coding region of nsP3 and was used in combination with 0220. 0113

10 was used to remove an *ApaI* endonuclease restriction site within the structural genes. 0140 encodes a unique NotI endonuclease restriction site followed by a 12 T's, and the reverse complement of the last 21 nucleotides of the VEE IE. A *NotI* site at the end of

15 the cDNA encoding the VEE IE genome allowed for run-off transcription after digestion of the plasmid with *NotI*. 0220 introduces a *ClaI* endonuclease restriction site within the coding region of nsP3 and was used in combination with 0126. Minor alterations of the

20 nucleotide sequence using 0077, 0113, 0126, and 0220 facilitated assembly of the full-length clones and allowed for rapid diagnostic analysis of virus generated from these clones by RT/PCR methods.

The full-length clone obtained, pIE1006, was

25 transcribed *in vitro* using T7 polymerase of *NotI*-linearized plasmid and the RNA transfected into BHK-21 cells (Figure 4). The phenotype of the resulting virus was markedly different from the parent virus (strain 68U201) from which the cDNA was derived. The

30 virus derived from pIE1006 *in vitro* transcribed RNA, VIE1006, gave rise to small plaques upon infection of target monolayers (Table 9). In attempts to recover the phenotypic characteristics of strain 68U201, regions of the pIE1006 clone were replaced with cDNA

35 generated by RT-PCR from strain 68U201 RNA. Three

44

subsequent full-length, live clones were constructed
by replacing pIE1006 sequences with 928, 4492, and
8130 nucleotides generated by RT-PCR of strain 68U201
RNA. These clones were designated pIE1007, pIE1008
5   and pIE1009; and viruses derived from these clones
were designated VIE1007, VIE1008 and VIE1009,
respectively. These clones are shown in Figure 4. The
relevant cloning sites and genetic markers are
indicated above each clone. Numbers indicate the
10   corresponding nucleotides in the strain 68U201 genome.
The T7 promoter is shown. Drawings are not to scale.
Shaded areas represent regions of pIE1006 that were
replaced or mutated to generate new clones. The
characteristics of these viruses are described below.
15

EXAMPLE 6

Assessment of Viruses Derived from Various
Clones. The viruses VIE1006, VIE1007, VIE1008 and
VIE1009 were derived from plasmids pIE1006, pIE1007,
20   pIE1008, and pIE1009, respectively. Analysis of virus
derived from these clones by plaque assay is shown in
Table 9. Plaque size was determined by infection of
Vero cell monolayers followed by agarose overlay.
    Analysis of virus derived from these clones
25   (Table 9) by plaque assay indicated that VIE1007 gave
the same plaque morphology as VIE1006 and therefore
was not studied further. The VIE1008 produced larger
plaques in comparison to VIE1006, and the VIE1009
virus gave rise to the largest sized plaques of the
30   four viruses tested. Plaques generated by VIE1009
were similar to those produced by the parental virus,
strain 68U201.

35

Table 9: In vitro analysis of virus derived from
molecular clones of VEE IE strain 68U201

Virus Strain[1]	Plaque Size
VIE1006	small
VIE1007	small
VIE1008	medium
VIE1009	large
VEE IE 68U201	large

EXAMPLE 7

In Vivo studies of VEE IE Vaccine Candidates. The
distinct plaque morphologies of VIE1006, VIE1008, and
VIE1009 suggested that these viruses may behave
differently *in vivo*. To assess the relative virulence
of the cloned derivatives of strain 68U201, mice were
initially inoculated with subcutaneously VIE1006,
VIE1008, and VIE1009 viruses (Table 10). Mice
infected with VIE1006 and VIE1008 were not adversely
affected by these viruses as assayed by the number of
mice surviving the infection. VIE1009 proved to be as
virulent as strain 68U201, causing death in all of the
animals infected (Table 10). Immunogenicity of the
different viruses inferred by the demonstration of a
protective immune response and was determined by back
challenge of surviving animals in the virulence assay
with approximately 10^4 pfu of the virulent, parental
virus, strain 68U201. Back challenge was performed
four weeks after the initial inoculation.

46

Table 10: In vivo analysis of virus derived from
molecular clones of VEE IE strain 68U201

5 **Virus**
    Strain[1]   **Mortality[2]**   **Challenge[3]**
    VIE1006   0/10   10/10
    VIE1008   0/10   10/10
    VIE1009   10/10   nd
10 **VEE IE 68U201** nd   1/10

[1] Initial inoculation with approximately 10^4 PFU of
each virus with the exception of VEE IE 68U201 which
were left untreated until challenge phase of
15 experiment.

[2] Expressed as animals dying/animals tested.

[3] Expressed as animals surviving/animals tested. All
animals were challenged with approximately 10^4 PFU of
VEE IE strain 68U201 four weeks after the initial
20 inoculation.

                        EXAMPLE 8

          Construction of a Full-length, Molecularly

    Attenuated VEE IE Clone. With the availability of a
25 full-length virulent clone, specific attenuating
    mutations were introduced into the structural genes of
    the virus by site-directed mutagenesis. A deletion
    mutation was used instead of a point mutation because
    of the inability of viral RNA replication to repair
30 such mutations. The glycoproteins of VEE IE are
    produced as a poly-protein precursor, PE2. The
    junction between the E3 and E2 proteins is cleaved by
    a furin-like cellular protease. The amino acid
    sequence of the presumed furin-like protease cleavage
35 site of strain 68U201 is RGKR. The
    nucleotides encoding these four amino acids of the
    furin-like cleavage site between the E3 and E2

proteins were deleted from the pIE1009 clone and the resulting "cleavage deletion" clone was designated pIE1100. The oligonucleotides used to generate the cleavage deletion mutation are shown in Table 8.

5   Oligonucleotide 0136 encodes a cleavage deletion mutation that eliminates the four amino acid furin-like cleavage site found in PE2 and was used in combination with oligonucleotide 0137. 0137 encodes the reverse complement of 0136.

10   Transfection of RNA transcribed from pIE1100 into tissue culture cells required extended incubation periods before viral cytopathic effect became apparent in the cultured cells. This extended incubation period is indicative of transcripts from full-length

15   clones possessing mutations that partially inhibit viral replication. However, such mutations can be suppressed by second-site mutations which arise randomnly via the error prone process of alphavirus replication, resulting in variants with enhanced

20   replication ability. In fact, the culture media from cells transfected with RNA transcribed from pIE1100 contained low titers of infectious virus, VIE1100, which could be amplified to high titer (4×10^7 PFU/ml) upon subsequent passage. Biochemical analysis showed

25   that this virus had an uncleaved PE2 protein indicating that the cleavage deletion mutation totally prevented proteolytic processing of the surface E2 glycoprotein precursor. The efficacy of VIE1100 virus to serve as a vaccine was evaluated in mice as

30   described below.

One specific suppressor mutation is thought to reside at nucleotide 10,181 of the VEE IE genome, a C to U nucleotide substitution resulting in an amino acid change from Serine to Leucine in the E1 protein

35   positon #57. Ability of this mutation to suppress the

lethal nature of the furin cleavage site deletion
mutation was assessed. The cDNA of pIE1100 was
mutated by changing nucleotide 10,181 from a C to T.
This introduced a serine to leucine change at amino

5   acid number 57 of E1, a mutation found within the
stock of VIE1100. The resulting clone was designated
pVIE1150. Transfection of RNA from pVIE1150 into BHK-
21 cells lead to the production of approximately 10^6
PFU/ml of virus in the supernatant of the transfected

10 cells at 48 hours post-transfection.

EXAMPLE 9

Vaccine Studies with a Full-length, Molecularly
Attenuated Virus. Balb/C mice were inoculated with

15 VIE1100 virus at various doses (10^4, 10^6, and 10^7 PFU
per mouse), and was found to be completely attenuated
at a dose 100,000 times higher than that required to
cause lethal disease by the parent virus, strain
68U201 (Table 10). Subsequent challenge of these

20 animals with virulent strain 68U201 demonstrated that
immunization with VIE1100 virus provided complete
protection from lethal virus challenge (Table 11).

Table 11:Vaccination study with VIE1100

25

Virus Strain Dose	Mortality[1]	Challenge[2]
VIE1100		
10^4	10/10	10/10
10^6	10/10	10/10
10^7	10/10	10/10
Mock Vaccinated	0/10	0/1C

35 1. Expressed as animals dying/animals tested.

2. Expressed as animals surviving/animals tested
after inoculation with 10^4 PFU of VEE IE strain 68U201

49

EXAMPLE 10

Properties of a WEE-EEE chimeric virus. Based
upon similarities of amino acid sequences of the
carboxy terminal portion of NSP-4 and the amino
5   terminal portion of the capsid proteins of WEE, EEE
and Sindbis virus, it has been suggested that WEE
virus arose by recombination between EEE and a Sindbis
virus ancestor (Hahn et al. [1988] Proc. Natl. Acad.
Sci. U.S.A. 85: 5997-6001). The capsid genes of both
10 WEE and EEE contain a highly conserved sequence with a
unique Bln I site 76 nucleotides downstream of the
initiation codon. We utilized this site to insert the
structural protein sequences of EEE into pWE5-18 in
order to construct a full length clone encoding a
15 chimeric virus. Plasmid pMWE-7 is a full length clone
consisting of the 5' non-coding sequence,
nonstructural genes, 26S promoter and the first 25
codons for the capsid protein of WEE CBA/87 fused to
the structural protein genes and 3' non-coding
20 sequence of EEE strain Fla91-4679.   Transfection of
BHK cells with RNA transcribed from pMWE-7 resulted in
complete destruction of the monolayer and release of
high yields of virus. SDS-polyacrylamide gel
electrophoresis of the purified virus demonstrated
25 that the virus is composed of polypeptides which
comigrate with those of EEE and not of WEE, indicating
that the virus is a chimera (Figure 3).

        Injection of mice with the MWE chimeric virus
killed mice only sporadically, suggesting that fusion
30 of the sequences of the two viruses resulted in
significant attenuation compared to the parent WE2000
virus (Table 12).   Mice immunized with MWE-7
developed significant neutralizing antibody titers and
resisted a lethal EEE Fla91-1467 challenge when
35 immunized with greater than 10^5 plaque forming units of

50

MWE-7 virus. The neutralizing antibody response after
challenge was not significantly elevated indicating
that the immunization with the chimeric virus
effectively prevented infection by a lethal EEE
5   challenge. Therefore, a chimeric virus derived by
combining the structural protein genes of EEE with the
non-structural proteins genes of WEE may serve as a
safe, effective approach to development of a vaccine
for EEE virus.

10

Table 12. Immunization of C57BL6 Mice with an WEE/EEE
Chimeric Virus Confers Protection Against a Lethal EEE
Challenge

Virus Strain Dose	Mortality[1]	Mean Day to Death	Challenge[2]
WE2000			
10^5	10/10 (100)	8.5	
MWE7			
10^3	0/10 (0)		6/10 (60)
10^5	1/10 (10)	8.0	9/9 (100)
10^7	0/10 (0)[3]		9/9 (100)
Saline Control	0/10 (0)		0/10 (0)

15 1. Expressed as animals dying/animals tested
    2. Expressed as animals surving/animals tested
    3. One animal died during pre-challenge bleed

20   **Example 12**
                         VEE IIIA Chimera

Construction of IAB-IIIA cDNA Chimeric Clone
(pV3A-1000)
25   BeAn8 (wild type Mucambo or VEE IIIA) cDNA was
amplified with primers 128 and 140 (Table 13) to
generate a PCR fragment of the entire 26S region of
BeAn8. Primer 128 incorporated an ApaI site just
upstream of the BeAn8 26S promoter to facilitate
30 cloning into pV3000. Primer 140 introduced a NotI

site just downstream of the poly(A) tract which would also facilitate cloning into pV3000 as well as enable run-off transcription. This 128/140 PCR fragment was cloned into pBluescript SK+ vector using the ApaI and

5   NotI sites and was termed pMUC-1000.

To replace the BeAn8 3' nontranslated region (3'NTR) in pMUC-1000 with the corresponding region in pV3000, an EcoRI site was introduced immediately downstream of the E1 stop codon in both pMUC-1000 and

10   pV3000 using the primer pairs 013/227 and 225/226, respectively. The 013 primer is a universal primer found in the pBluescript SK+ vector and the 227 primer was designed to introduce an EcoRI site in pMUC-1000. The 225 primer introduced an EcoRI site in pV3000

15   and the 226 primer is located in the vector, TotoX. The 013/227 and 225/226 PCR fragments were separately cloned into the pBluescript SK+ vector producing pMUC-1200 and pV3nt-1000, respectively. The 3' NTR encoded in pV3nt-1000 was shuttled into pMUC-1200 using the

20   EcoRI site and the NotI site to produce pMUC-1300.

The full-length chimeric cDNA clone, pV3A-1000, was constructed by shuttling the structural genes encoded in pMUC-1300 into the pV3000 nonstructural

25   domain using the ApaI site and NotI site.

Table 13. Primers for construction of the pV3A-1000
013 -- AACAGCTATGACCATG (SEQ ID NO:24)
128 -- CTGAGAGGGGCCCCAGTAAC (SEQ ID NO:25)
30   140 -- GACTGCGGCCGCTTTTTTTTTTTTTGAAATATTAAAAA (SEQ ID
          NO: 26)
225 -- CCAGAAACATAATTGAATTCAGCAGCAATTG (SEQ ID NO:27)
226 -- CTTTATCCGCCTCCATCC (SEQ ID NO:28)
227 -- CCAATCGCTGCTGAATTCTAATTATGTTTCTG (SEQ ID NO:29)
35

Five week old C57BL6 mice were used in a challenge study (Table 14). All mice immunized with

52

10^7 PFU of pV3A-1000 produced neutralizing antibodies.
Mice were challenged with 10^7 PFU of 900807 (a Mucambo
virus from Trinidad). The geometric mean neutralizing
antibody titer in mice inoculated with 10^7, 10^5, and

5   10^3 PFU of pV3A-1000 was 9800, 8800, and 7000
respectively.

Table 14. Results from challenge studies

	Fraction of mice sick/inoc	Fraction of mice dead/inoc	Average days to onset of illness	Average days to death
Immunized with pV3A-1000	0/20	0/20	N/A	N/A
Unvaccinated	19/20	12/20	6.2	10.5

53

SEQUENCE LISTING

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                           Jonathan F. Smith
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10

            (ii) TITLE OF INVENTION: Live Attenuated virus
       vaccines for western equine encephalitis virus,
       eastern equine encephalitis virus, and venezuelan
         equine encephalitis virus IE and IIIA variants

15

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            (v) COMPUTER READABLE FORM:
                  (A) MEDIUM TYPE: Floppy disk
                  (B) COMPUTER: Apple Macintosh
                  (C) OPERATING SYSTEM: Macintosh 7.5
30   (D) SOFTWARE: Microsoft Word 6.0

            (vi) CURRENT APPLICATION DATA:
                  (A) APPLICATION NUMBER:
                  (B) FILING DATE:
35   (C) CLASSIFICATION:

            (vii) PRIOR APPLICATION DATA:
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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 11492 base pairs
        (B) TYPE: Nucleic acid
        (C) STRANDEDNESS:Double
        (D) TOPOLOGY: Linear

    (ii) SEQUENCE DESCRIPTION: SEQ ID NO:1:

1 ATAGGGCATG GTATAGAGGC ACCTACCCTA CAAACAAATC
GATCCAATAT

      51 GGAAAGAATT CACGTTGACT TAGACGCTGA CAGCCCATAT
GTCAAGTCGT

     101 TACAGCGGAC GTTTCCACAA TTTGAGATCG AAGCAAGGCA
GGTCACTGAC

     151 AATGACCATG CCAATGCCAG AGCGTTTTCG CATGTGGCAA
CAAAGCTCAT

     201 TGAGAGCGAA GTCGACCGGG ACCAAGTTAT CTTGGACATT
GGAAGTGCGC

     251 CCGTCAGACA TGCACATTCC AACCACCGCT ATCATTGTAT
CTGCCCCATG

     301 ATAAGCGCTG AAGACCCGGA CAGACTACAG CGGTATGCAG
AAAGACTTAA

     351 GAGAAGTGAC ATGTACCGAC AAGAATATAG CCTCTNAAGG
CGGCAGACCT

     401 GCTGGAAGTC ATGTCCACAC CAGACGCAGA GACTCCATCT
CTGTGTATGC

     451 ACACAGACGC CACGTGTAGG TACTTTGGAA GTGTANGCAG
TATACCAAGA

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      501   TGTGTACGCA  GTCCATGCAC  CGACATCAAT  CTACCACCAG
   GCGCTTAAAG

      551   GAGTTAGGAC  AATTTACTGG  ATAGGTTTTG  ACACGACCCC
 5 TTTTATGTAC

      601   AAAAACATGG  CAGGTTCCTA  CCCTACTTAC  AACACAAACT
   GGGCCGACGA

10       651   GAGAGTATTG  GAAGCACGTA  ACATTGGCCT  CGGTAACTCA
   GATCTTCAGG

      701   AGAGCAGACT  TGGAAAACTT  TCAATCCTTA  GGAAGAAGAG
   GCTCCAACCT
15
      751   ACTGATAAGA  TCATATTCTC  GGTTGGTTCA  ACAATCTACA
   CAGAGGATAG

      801   ATCACTGTTA  CGTAGCTGGC  ATCTTCCAAA  CGTGTTCCAC
20 CTGAAAGGAA

      851   AGTCTNACTT  CACAGGTAGA  TGTGGGACCA  TTGTCAGCTG
   TGAAGGGTAT

25       901   GTCATCAAAA  AGATAACGAT  CAGCCCAGGA  CTATACGGTA
   AAGTTGAGAA

      951   CTTGGCGTCC  ACGATGCATC  GCGAGGGTTT  CTTGAGTTGC
   AAAGTCACAG
30
     1001   ATACGCTGCG  CGGCGAGAGG  GTTTCTTTTG  CTGTGTGTAC
   GTATGTACCA

     1051   GCCACACTTT  GCGATCAGAT  GACAGGGAGG  GCTCAACCAA
35 CGGATTGTCG

     1101   TCAATGGTAG  GACGCAAAGA  AATACTNACA  CAATGCAGAA
   CTATCTATTA

40      1151   CCAGTGGTCG  CCCAGGCGTT  TTCCAGGTGG  GCGCGTGNAC
   ATCGTGCCGA

     1201   CTTGGACGAC  GAGAAGGAGC  TAGGGGTGCG  GGAGCGCACT
   CTTACTATGG
45
     1251   GCTGCTGCTG  GGCTTTCAAG  ACCCAGAAAA  TTACATCCAT
   CTACAAGAAG

     1301   CCTGGTACGC  AAACAAATTA  AGAAAGTACC  TGCCGTCTTT
50 TGACTCATTT
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1351  GTGATTCCGA CGCCTTACCA GCCACGCGGG GGCTCGAATA
TGGGCCTTCC

1401  GCCGTNAGGC TCAAGCTGCT GCTTGAACCA ACTGTCAAAC
CCGCACCGGC

1451  TATTACAATG GCCGATGTGG AGCACCTGCG TGGCTTACAG
CAAGAAGCTG

1501  AAGAAGTGGC TGCAGCGGGA AGAGATCAGA GAAGCCCTGC
CACCCTTGCT

1551  CCCTGAAATA GAAAAAGAGA CCGTAGAGGC AGAAGTAGAC
CTCATTATGC

1601  AAGAGGCAGG AGCAGGTAGC GTGGAGACAC CNACGAGGAC
ATATCAAGGT

1651  AACAAGTTAC CCAGGTGNAA GAGAAGATTG GGTCTTATCC
CTATACTTTC

1701  ACCCCAGGCG GTTTTANAAT NGTNAAAAAC TGGCGTGTAT
CCACCCATTG

1751  GCGGAACAAG TACTGGTAAT GACTCACAAA GGCAGGGCCG
GGCGATACAA

1801  AGTCGAGCCA TACCACGGTA NGGTCATTGT ACCCAGAAGG
GACGGCGGGT

1851  CCCTGTTCAA GACTTTCAGG CACTGAGTGA GAGCGCCACG
ATCGTTTTCA

1901  ACGAGAGGGA GTTCGTAAAC CAGATATTTT GCACCCACAT
CGCAAGCKTT

1951  TCAACTATAG TGAGTCGCTA TTACACTGAC GAAGAGTACT
ATAAGATTGT

2001  AAAGACTCAG GACGCAGACT CAGAATACGT CTTTGACATT
GACGCACGAA

2051  AGTGTGTTAA GCGAGAAGAC GCAGGTCCCT TGTGCCTAAC
TGGTGATCTG

2101  GTAGATCCAC CATTTCACGA GTTTGCGTAC GAGAGTCTCA
AGACACGACC

2151  AGCAGCACCT CACAAAGTCC CAACCATCGG AGTCTATGGA
GTGCCAGGTT
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57

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    2201   CAGGTAAATC TGGGATCATC AAAAGCGCTG TGACTAAGAA
       AGATCTGGTT

    2251   GTGAGTGCGA AGAAGGAAAA CTGCGCAGAA ATTATCAGGG
 5     ATGTAAGGAG

    2301   GATGAGACGT ATGGATGTTG CTGCTAGGAC TGTTGATTCA
       GTGCTTCTAA

10     2351   ATGGGGTTAA GCACCCCGTT AACACCCTGT ACATTGATGA
       GGCATTTGCC

    2401   TGCCATGCAG GGACGCTGCT GGCACTGATT GCCATCGTCA
       AACCTAAGAA
15
    2451   AGTGGTATTG TGCGGGGACC CAAAACAATG CGGCTTCTTT
       AACATGATGT

    2501   GCCTGAAAGT ACATTTTAAC CATGACATAT GCACTGAGGT
20     GTACCACAAA

    2551   AGCATCTCTA GGAGGTGCAC ACAGACTGTA ACCGCCATTG
       TCTCCACGCT

25     2601   CTTCTACGAC AAGCGAATGA AGACGGTTAA CCCATGTGCT
       GACAAAATCA

    2651   TCATAGATAC CACAGGGACC ACAAAGCCGC ACAAAGATGA
       TCTGATTCTA
30
    2701   ACCTGTTTCA GAGGATGGGT GAAACAGCTA CAGATTGACT
       ATAAAAATCA

    2751   TGAAATCATG ACTGCGGCTG CATCGCAAGG ACTTACGCGG
35     AAAGGCGTTT

    2801   ATGCTGTCAG GTACAAAGTC AACGAGAATC CACTCTACTC
       GCAGACTTCT

40     2851   GAGCACGTGA ACGTGTTAGT TACACGCACA GAAAAACGCA
       TTGTCTGGAA

    2901   GACGCTAGCT GGTGACCCCT GGATAAAGAC ACTTACAGCT
       AAATACTCCG
45
    2951   GGGATTTCAC GGCTTCATTG GACGACTGGC AACGCGAACA
       CGACGCTATT

    3001   ATGGCACGCG TTCTTGATAA GCCGCAGACA GCTGATGTGT
50     TCCAGAATAA
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58

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   3051  GGTGAACGTC TGCTGGGCGA AGGCTTTAGA GCCAGTCTTG
GCCACGGCCA

   3101  ACATTGTGCT GACGAGACAG CAGTGGGAGA CGTTGCACCC
ATTCAAGCAT

   3151  GACAGAGCGT ACTCACCTGA AATGGCACTG AACTTCTTTT
GCACCAGGTT

   3201  CTTTGGTGTA GACCTGGACA GTGGGTTGTT TTCCGCTCCT
ACCGTCGCAC

   3251  TTACTTACAG GGATCAGCAC TGGGACAACT CGCCAGGGAA
GAACATGTAT

   3301  GGGCTTAATA GAGAGGTAGC AAAGGAGCTG TCACGGCGAT
ATCCGTGCAT

   3351  CACAAAAGCG GTTGACACAG GCAGGGTAGC TGATATAAGG
AATAATACCA

   3401  TCAAGGACTA CTCTCCAACA ATTAATGTGG TTCCATTAAA
TCGCCGGTTG

   3451  CCCCACTCGC TGATCGTTGA CCACAAAGGA CAGGGTACAA
CTGATCACAG

   3501  TGGATTCCTA TCTAAGATGA ATGGCAAATC TGTGTTGGTG
ATCGGCGATC

   3551  CTATCAGCAT TCCTGGGAAG AAAGTAGAGT CCATGGGTCC
ATTGCCCACT

   3601  AATACCATCA GGTGTGATCT CGATTTGGGA ATACCTAGCC
ATGTCGGTAA

   3651  ATATGACATT ATATTTGTCA ATGTTAGGAC CCCATACAAG
AACCATCACT

   3701  ACCAACAGTG CGAGGATCAC GCTATCCACC ACAGCATGCT
AACGTGTAAG

   3751  GCTGTCCACC ACCTGAACAC TGGCGGAACA TGTGTGGCCA
TAGGGTATGG

   3801  ACTTGCTGAT CGCGCAACCG AGAATATCAT CACTGCGGTG
GCTCGCTCAT

   3851  TTAGGTTTAC CCGTGTCTGT CAGCCTAAGA ACACTGCCGA
AAATACTGAG
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    3901  GTTCTCTTCG TGTTCTTCGG CAAGGACAAC GGCAACCACA
CACATGACCA

    3951  GGACAGACTC GGTGTAGTGC TTGACAACAT CTACCAAGGG
TCAACCAGGT

    4001  ACGAGGCAGG GAGAGCTCCA GCGTACAGAG TGATCAGAGG
TGACATTAGC

    4051  AAGAGCGCTG ACCAAGCTAT CGTTAATGCT GCTAATAGCA
AAGGTCAACC

    4101  AGGTTCCGGA GTGTGCGGTG CACTGTACCG AAAATGGCCG
GCTGCTTTTG

    4151  ATAGACAGCC AATAGCTGTC GGGACGGCTA GACTTGTGAA
GCACGAACCG

    4201  CTCATCATAC ATGCTGTAGG ACCGAATTTT TCTAAGATGC
CGGAACGAGA

    4251  GGGCGACCTT AAGCTCGCAG CTGCCTACAT GAGCATAGCG
TCCATCGTCA

    4301  ACGCTGAGCG GATTACTAAA ATATCAGTAC CGCTACTGTC
AACTGGCATC

    4351  TATTCTGGTG GCAAAGATCG AGTGATGCAA TCATTGCATC
ACTTGTTCAC

    4401  TGCTTTCGAC ACTACGGATG CCGATGTCAC CATATATTGC
TTGGATAAAC

    4451  AATGGGAGAC CAGGATAACC GAGGCCATTC ACCGCAAAGA
AAGCGTCGAA

    4501  ATACTGGATG ATGATGACAA GCCAGTAGAC ATCGACTTGG
TCAGGGTCCA

    4551  CCCAAACAGC TCTTTGGCAG GCAGACCAGG TTACTCCGTC
AATGAGGGCA

    4601  AGCTGTATTC ATACCTGGAA GGTACACGAT TCCATCAGAC
CGCCAAGGAC

    4651  ATTGCCGAAA TCCATGCAAT GTGGCCCAAC AAATCTGAGG
CTAATGAGCA

    4701  GATTTGCTTG TACATCCTGG GGGAGAGTAT GTCCAGCATC
CGCTCCAAAT
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60

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    4751  GCCCAGTAGA GGAGTCAGAG GCGTCTGCTC CACCTCACAC
ACTTCCATGC

    4801  CTGTGTAATT ACGCTATGAC GGCTGAGCGC GTATACAGGT
TGCGCTCTGC

    4851  GAAGAAAGAA CAGTTCGCCG TATGCTCATC ATTCCCGTTG
CCGAAGTACA

    4901  GGATCACAGG CGTGCAGAAG CTACAGTGCA GCAAACCAGT
CCTGTTCTCA

    4951  GGCGTCGTAC CACCGGCTGT ACACCCCAGG AAGTACGCGG
AAATAATTCT

    5001  AGAAACGCCA CCATCGCCAA CAACGACAAC CGTAATATGT
GAACCAACTG

    5051  TGCCAGAACG TATACCCAGT CCGGTGATTT CTAGAGCAGC
AAGTGCGGAA

    5101  TCACTGCTAT CGTTTGGCGG CGTCTCGTTC TCTAGCTCTG
CCACACGCTC

    5151  GTCAACTGCC TGGAGCGACT ATGACAGGCG GTTTGTGGTT
ACAGCTGACG

    5201  TGCATCAAGC GGACACGTCT ACGTGGAGCA TCCCTAGCGC
TCCTGACTTG

    5251  GACGTCCAGC TGCCTTCTGA CGATACTGAT TCCCACTGGA
GTATTCCGAG

    5301  TGCATCAGGC TTTGAAGTGA GAACACCGTC TGTACAGGAC
CTAACTGCAG

    5351  AGTGTGCGAG GCCTCGTGGA CTGGCCGAAA TAATGCAAGA
CTTCAATACG

    5401  GCTCCTTTCC AGTTTCTTTC GGACCACAGA CCAGTACCAG
CACCACGGAG

    5451  ACGCCCCATC CCATCACCTA GATCGACGGT TTCCGCACCT
CCAGTTCCAA

    5501  AGCCACGCAG AACTAAGTAC CAACAACCAC CAGGAGTCGC
TAGGGCGATC

    5551  TCAGAAGCGG AGCTGGACGA GTACATCCGT CAACATTCCA
ATTGACGGTA
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61

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      5601   TGAAGCGGAG CGTATATTTC TCATCGAAAC AGGCCAGGTC
    ACCTTCAACA

      5651   GAAATCAGTA CGTCAATGTA AACTACAAGA CCCTATATTG
 5  GAACGGGCCG

      5701   TCCATGAGAA GTATTAGCCC CGCGCCTCGA TCTCGAAAGA
    GAGAAGATGT

10    5751   TACAGAAGAA ACTGCAATTA TGCGCCTCNT GAAGGAAATA
    GAAGCAGGTA

      5801   TCAATCACGA AAAGTANGAA AATATGAAAG CAATTACAGC
    GGAGCGACTC
15
      5851   ATTTCTGGAT TGGGCACATA TCTATCATCA GAAGTGAATC
    CTGTCGAGTG

      5901   TTACAGAGTC AATTATCCTG TACCAATCTA CTCGTCAACG
20  GTAGTTAATA

      5951   GGTTTTCATC AGCAGAAGTG GCCGTCAAAG TTTGCAACTT
    AGTCATCCAA

25    6001   GAGAATTACC CTACAGTAGC CAGTTATTGC ATAACAGATG
    AATACGATGC

      6051   GTATCTTGAC ATGGTGTACG GCGCTCGTGC TGTTAGATAC
    AGCGCCTTTG
30
      6101   TCCGCTNACT GAGAAGCTAC CCAAAGAAGC ATAGCTACTT
    GCAGCCAGAG

      6151   ATAAGATCAG CTGTCCCATC ACCTATACAG AATACATTAC
35  AAAATGTATT

      6201   GCTGCAGCCA CTAAAAGGAA CTGCAACGTT ACCCAAATGC
    GAGAACTACC

40    6251   TGTTTTATTC GGCGGCATTC AACGTTGAAT GTTTCAAGAA
    ATACGCATGC

      6301   AATGATGAGT ATTGGGATAC CTTTCGCGAT AACCCTATTC
    GGCTAACTAC
45
      6351   AGAGAACGTT ACGCAATACG TGACAAAGCT GAAAGGGCCG
    AAAGCAGCAG

      6401   CATTATTCGC AAAAACTCAC AACCTAAAAC CGTTGCAGGA
50  GATACCAATG
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      6451  GACGAATTTG TCATGGTCTN AAAAGAGATG TCAAAGTTAC
   TCCCGGCACA

      6501  AAACATACAG AGGAGCGGCC TAAGGTGCAG GTTATTCAGG
5  CTGCAGATCC

      6551  TCTCTGTACC GCTTACCTTT GCGGGATCCA TCGAGAATGT
   CCGTAGACTG

10      6601  AATGCTGTGC TTCTGCCGAA TATCCATACT CTCTTCGACA
   TGTCAGCGGA

      6651  AGATTTTGAT GCGATTATTG CTGAACATTT CCACCACGGC
   GACCCAGTAT
15
      6701  TGGAAACGGA CATCGCGTCG TTTGATAAAA GCGAAGACGA
   CGCTATCGCC

      6751  ATTTCGGCGT TGATGATCCT CGAGGACTTA GGCGTCGACC
20  AACCGCTCTT

      6801  AGATTTGATA GAGGCGGCGT TCGGCAATAT CACATCTGTG
   CACCTACCTA

25      6851  CAGGAACGAG GTTCAAATTT GGTGCCATGA TGAAATCCGG
   TATGTTCTTA

      6901  ACGCTGTTTG TCAACACACT AGTCAATATC ATGATTGCTA
   GCAGAGTACT
30
      6951  ACGTGAACGG TTAACCACGT CAGAGTGCGC GGCCTTATCG
   GCGACAATAA

      7001  CATAGTGCAC GGTGTCGCTC CGACAACTTG ATGGCGGAGA
35  GATGCGCCAC

      7051  TTGGCTGAAC ATGGAAGTAA AAATTATTGA TGCAGTCATT
   GGTATCAAAG

40      7101  CACCCTACTT CTGCGGGGAT TCATCCTGGT GGATCAGATA
   ACGACACAGC

      7151  CTGTAGGTCG CAGACCCTCT AAAAAGGCTT TTTAAGCTTG
   GAAAACCATT
45
      7201  GCCAGTCGAT GACACCCAAG ACTGTGACCG TCGCCGGGCA
   CTGCATGATG

      7251  AAGCAATGCG ATGGAACAGA ATTGGAATTA CGGACGAGTT
50  GGTGAAGGCC
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63

7301   GTAGAATCCA GATACGAGAT CATACTGGCA GGCCTGATCA
TCACGTCCCT

7351   GTCCACGTTA GCCGAAAGCG TTAAGAACTT CAAGAGCATA
AGAGGGAACC

7401   CAATCACCCT CTACGGCTGA CCTAAATAGG TGACGTAGTA
GACACGCACC

7451   TACCCACCGC CAAAAATGTT TCCATACCCT CAGCTGAACT
TTCCACCAGT

7501   TTACCCTACA AATCCGATGG CTTACCGAGA TCCAAACCCT
CCTAGGCGCC

7551   GCTGGAGGCC GTTTCGGCCC CCGCTGGCTG CTCAAATCGA
AGATCTTAGG

7601   AGGTCGATAG CCAACTTAAC TTTCAAACAA CGATCACCTA
ATCCGCCGCC

7651   AGGTCCGCCG CCGAAGAAGA AGAAGAGTGC TCCCAAGCCA
AAACCTACTC

7701   AGCCTAAAAA GAAGAAGCAG CAAGCCAACA AGACGAAACG
CAAGCCTAAA

7751   CCAGGGAAAC GACAGCGTAT GTGTATGAAG TTGGAGTCGG
ACAAGACATT

7801   TCCGATCATG TTGAACGGCC AAGTGAATGG ATACGCTTGC
GTTGTCGGAG

7851   GAAGGCTGAT GAAACCACTC CACGTTGAAG GAAAAATCGA
TAATGAGCAA

7901   TTAGCGGCCG TGAAATTGAA GAAGGCTAGC ATGTACGACC
TGGAGTATGG

7951   CGACGTTCCC CAGAATATGA AATCAGACAC GCTGCAGTAC
ACCAGCGACA

8001   AACCACCGGG CTTCTACAAC TGGCACCACG GCGCAGTCCA
GTATGAGAAT

8051   GGGAGATTCA CCGTACCGCG AGGAGTGGGC GGGAAAGGCG
ACAGTGGAAG

8101   ACCGATCCTG GACAACAGAG GCAGAGTTGT GGCTATTGTT
CTAGGAGGTG

64

8151   CAAACGAGGG CACGCGTACG GCGCTTTCAG TGGTCACTTG
GAACCAGAAA

5   8201   GGGGTGACCA TCAAGGATAC CCCCGAAGGT TCTGAACCGT
GGTCACTAGT

8251   TACAGCGCTG TGCGTGCTTT CGAATGTCAC ATTCCCTTGC
GACAAACCAC

10   8301   CCGTCTGCTA TTCACTGGCG CCAGAACGAA CACTCGACGT
GCTCGAGGAG

8351   AACGTCGACA ATCCAAATTA CGACACGCTG CTGGAGAACG
TCTTGAAATG
15

8401   TCCATCACGC CGGCCCAAAC GAAGCATTAC CGATGACTTC
ACGCTGACCA

8451   GTCCCTACCT GGGGTTCTGC CCGTATTGCA GACACTCAGC
20   GCCATGTTTT

8501   AGCCCAATAA AAATTGAGAA CGTGTGGGAC GAATCTGATG
ATGGGTCGAT

25   8551   TAGAATCCAG GTCTCGGCAC AATTCGGCTA CAATCAGGCA
GGCACTGCAG

8601   ACGTCACCAA GTTCCGGTAC ATGTCTTACG ACCACGACCA
TGACATCAAG
30

8651   GAAGACAGTA TGGAGAAAAT AGCTATTAGT ACATCTGGAC
CATGCCGTCG

8701   TCTTGGCCAC AAAGGGTACT TCCTGTTAGC TCAATGTCCT
35   CCAGGTGACA

8751   GTGTAACCGT CAGTATCACG AGCGGAGCAT CTGAGAATTC
ATGCACCGTG

40   8801   GAGAAAAAGA TCAGGAGGAA GTTTGTCGGT AGAGAGGAGT
ACTTGTTCCC

8851   ACCTGTCCAT GGAAAGCTGG TAAAGTGCCA CGTTTACGAT
CACTTGAAGG
45

8901   AGACGTCTGC CGGATACATA ACCATGCACA GGCCAGGCCC
ACACGCGTAT

8951   AAGTCCTATC TGGAGGAAGC GTCAGGCGAA GTGTACATTA
50   AACCACCTTC

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     9001   TGGCAAGAAC GTCACCTACG AATGTAAGTG TGGTGACTAC
     AGCACAGGTA

     9051   TTGTGAGCAC GCGAACGAAG ATGAACGGCT GCACTAAAGC
 5   AAAACAATGC

     9101   ATTGCCTACA AGCGCGACCA AACGAAATGG GTCTTCAACT
     CGCCGGATCT

10   9151   TATTAGGCAC ACAGACCACT CAGTGCAAGG TAAACTGCAC
     ATTCCATTCC

     9201   GCTTGACACC GACAGTCTGC CCGGTTCCGT TAGCTCACAC
     GCCTACAGTC
15
     9251   ACGAAGTGGT TCAAAGGCAT CACCCTCCAC CTGACTGCAA
     CGCGACCAAC

     9301   ATTGCTGACA ACGAGAAAAT TGGGGCTGCG AGCAGACGCA
20   ACAGCAGAAT

     9351   GGATTACAGG GACTACATCC AGGAATTTTT CTGTGGGGCG
     AGAAGGGCTG

25   9401   GAGTACGTAT GGGGCAACCA TGAACCAGTC AGAGTCTGGG
     CCCAGGAGTC

     9451   GGCACCAGGC GACCCACATG GATGGCCGCA TGAGATCATC
     ATCCACTATT
30
     9501   ATCATCGGCA TCCAGTCTAC ACTGTCATTG TGCTGTGTGG
     TGTCGCTCTT

     9551   GCTATCCTGG TAGGCACTGC ATCGTCAGCA GCTTGTATCG
35   CCAAAGCAAG

     9601   AAGAGACTGC CTGACGCCAT ACGCGCTTGC ACCGAACGCA
     ACGGTACCCA

40   9651   CAGCATTAGC AGTTTTGTGC TGTATTCGGC CAACCAACGC
     TGAAACATTT

     9701   GGAGAAACTT TGAACCATCT GTGGTTTAAC AACCAACCGT
     TTCTCTGGGC
45
     9751   ACAGTTGTGC ATCCCTCTGG CAGCGCTTGT TATTCTGTTC
     CGCTGCTTTT

     9801   CATGCTGCAT GCCTTTTTTA TTGGTTGCAG GCGTCTGCCT
50   GGGGAAGGTA
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 9851   GACGCCTTCG  AACATGCGAC  CACTGTGCCA  AATGTTCCGG
GGATCCCGTA

 9901   TAAGGCGTTG  GTCGAACGTG  CAGGTTACGC  GCCACTTAAT
CTGGAGATTA

 9951   CGGTCGTCTC  ATCGGAATTA  ACACCCTCAA  CTAACAAGGA
GTACGTGACC

10001   TGCAAATTTC  ACACAGTCGT  TCCTTCACCA  CAAGTTAAAT
GCTGCGGGTC

10051   CCTCGAGTGT  AAGGCATCCT  CAAAAGCGGA  TTACACATGC
CGCGTTTTTG

10101   GCGGTGTGTA  CCCTTTCATG  TGGGGAGGCG  CACAGTGCTT
CTGTGACAGT

10151   GAGAACACAC  AACTGAGTGA  GGCATACGTC  GAGTTCGCTC
CAGACTGCAC

10201   TATAGATCAT  GCAGTCGCAC  TAAAAGTTCA  CACAGCTGCT
CTGAAAGTCG

10251   GCCTGCGTAT  AGTATACGGC  AATACCACAG  CGCGCCTGGA
TACATTCGTC

10301   AACGGCGTCA  CACCAGGTTC  CTCACGGGAC  CTGAAGGTCA
TAGCAGGGCC

10351   GATATCAGCA  GCTTTTTCAC  CCTTTGACCA  TAAGGTCGTC
ATTAGAAAGG

10401   GGCTTGTTTA  CAACTACGAC  TTCCCTGAGT  ATGGAGCTAT
GAACCCAGGA

10451   GCGTTCGGCG  ATATTCAAGC  ATCCTCTCTT  GATGCCACAG
ACATAGTAGC

10501   CCGCACCGAC  ATACGGCTGC  TGAAGCCTTC  TGTCAAGAAC
ATCCACGTCC

10551   CCTACACCCA  AGCAGTATCA  GGGTATGAAA  TGTGGAAGAA
CAACTCAGGA

10601   CGACCCCTGC  AAGAAACAGC  ACCATTCGGA  TGTAAAATTG
AAGTGGAGCC

10651   TCTGCGAGCG  ACTAACTGTG  CTTATGGGCA  CATCCCTATC
TCGATTGACA
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67

10701   TCCCTGATGC AGCTTTTGTG AGATCATCTG AATCACCAAC
AATTTTAGAA

5   10751   GTCAGCTGCA CAGTAGCAGA CTGCATTTAT TCTGCAGACT
TTGGTGGTTC

10801   GCTAACACTA CAGTACAAAG CTAACAGAGA GGGACATTGT
CCAGTTCACT

10   10851   CCCACTCCAC TACAGCTGTT TTGAAGGAAG CGACCACACA
TGTGACTGCC

10901   ACAGCCATAA CACTACATTT TAGCACATCG AGCCCACAAG
CAAATTTCAT
15
10951   AGTTTCGCTA TGGCGCAAGA AGACCACCTG CAATGCTGAA
TGTAAACCAC

11001   CGGCCGACCA CATAATTGGA GAACCACATA AGGTCGACCA
20   AGAATTCCAG

11051   GCGGCAGTTT CCAAAACATC TTGGAACTGG CTGCTTGCAC
TGTTTGGGGG

25   11101   AGCATCATCC CTCATTGTTG TAGGACTTAT AGTGTTGGTC
TGCAGCTCTA

11151   TGCTTATAAA CACACGTAGA TGACTGAGCG CGGACACTGA
CATAGCGGTA
30
11201   AAAACTCGAT GTACTTCCGA GGAAGCGTGG TGCATAATGC
CACGCGCCGC

11251   TTGACACTAA AACTCGATGT ATTTCCGAGG AAGCACAGTG
35   CATAATGCTG

11301   TGCAGTGTCA CATTAATCGT ATATTACACT ACATATTAAC
AACACTATAT

40   11351   CACTTTTATG AGACTCACTA TGGGTTTCTA ATACACACTA
CACACATTTT

11401   ATTTAAAAAC ACTACACACA CTTTATAAAT TCTTTTATAA
TTTTTCTTTT
45
11451   GTTTTTTATT TTGTTTTTAA AATTTCAAAA AAAAAAAAAA
AA

50

(3) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 11464 base pairs
              (B) TYPE: Nucleic acid
              (C) STRANDEDNESS:Double
              (D) TOPOLOGY: Unknown
          1 ATGGGCGGCG CATGAGAGAA GCCCAAACCA ATAACTACCC
    AAAATGGAGA AAGTTCACGT

         61 TGACATCGAG GAAGATAGTC CCTTCCTCAG AGCATTACAA
    CGGAGCTTCC CGCAGTTTGA

        121 GGTAGAAGCC AAGCAGGTCA CAGATAATGA CCATGCTAAC
    GCCAGAGCGT TTTCGCATTT

        181 GGCATCGAAA TTGATCGAGA CGGAGGTGGA ACCATCCGAT
    ACGATCCTAG ACATTGGAAG

        241 TGCGCCTGCC CGCAGAATGT ATTCCAAGCA TAAGTACCAT
    TGCATCTGTC CGATGAAATG

        301 TGCAGAAGAT CCGGACAGAC TGTTTAAGTA TGCAGCCAAG
    CTGAAGAAGA ACTGTAAAGA

        361 GATTACAGAT AAGGAACTGG ACAAGAAGAT GAAGGAGCTT
    GCGGAAGTCA TGAGCGACCC

        421 TGATCTCGAA ACTGAAACGA TTTGCCTTCA CGACGATGAA
    ACCTGTCGAT TTGAGGGTCA

        481 AGTCGCAGTG TATCAGGATG TGTACGCGGT TGACGGACCG
    ACGAGCCTTT ACCATCAGGC

        541 CAACAAAGGG GTCAGAGTCG CCTATTGGAT AGGATTCGAC
    ACTACCCCTT TTATGTTTAA

        601 GAACCTGGCT GGAGCATATC CCTCCTATTC GACCAACTGG
    GCCGACGAGA CCGTGTTAAC

        661 GGCTCGTAAT ATAGGCTTGT GCAGCTCCGA TGTGATGGAG
    CGGTCACGTA GAGGGATGTC

        721 CATCCTCAGA AAGAAATTTT TAAAACCATC CAATAACGTC
    TTGTTCTCTG TAGGATCTAC

        781 CATCTACCAC GAGAAGCGAG ACTTACTAAG GAGTTGGCAC
    CTACCGTCCG TTTTTCACCT

841 ACGTGGTAAG CAGAATTACA CTTGTCGGTG TGAGACTATA
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76

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79

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15   11461 TTTC

(4) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:
20   (A) LENGTH: 4003 base pairs
                (B) TYPE: Nucleic acid
                (C) STRANDEDNESS:Double
                (D) TOPOLOGY: Unknown
        Nucleotide Sequence of the BeAn8 26S Subgenomic
25 cDNA containing last portion of NS4, the 26S promoter,
    the complete BeAn8 structural genes and a portion of
    the 3' noncoding region.

30

        1 CCCAGTAACT CTCTACGGCT GACCTGAATG GACTGTAACG
TAGTTCAGTC

        51 CGCAACCATG TTCCCTTACC AATCACCAAT GTTTCCAATG
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25    2351  ATTTATGGAA CCATAATCAA CAGATGTTCT GGAGTCAACT
     GCTCATTCCC

      2401  CTAGCCGCGC TCATTGTGGT GACCCGCTTG CTGAAGTGCA
     TGTGTTGCGT
30
      2451  CGTTCCTTTT TTAGTCCTAG CAGGCGCCGC AAGCGTCGGC
     GCCTACGAAC

      2501  ACGCAACCAC GATGCCGAAT CAGGTGGGGA TCCCGTATAA
35   TACAGTAGTC

      2551  AACCGCGCAG GTGACGCACC ATTGGCAATC AGCATTATTC
     CAACCAAGAT

40    2601  ACGGCTAATT CCTACTTTGA ATTTAGAATA TATTACATGC
     CACTATAAGA

      2651  CAGGATTAGA TTCACCTTTC ATTAAATGCT GCGGAACGCA
     GGAATGCCCC
45
      2701  CAAGTGAATA GACCCGATGA ACAGTGTAAA GTCTTCACGG
     GGGTGTATCC

      2751  GTTTATGTGG GGAGGCGCCT ACTGCTTCTG TGACTCTGAA
50   AACACGCAAA
```

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2801  TTAGTCGAGC GTATGTGATG AAATCAGATG ACTGCTCAGC
TGACCACGCC

2851  TTGGCCTACA AAGCTCATAC TGCCTCAGTC CAAGCTTTTC
TGAATATAAC

2901  TGTGGGAGAG CAATCGACGA CAGCGGTAGT GTACGTGAAT
GGAGAAACAC

2951  CGGTCAATTT TAACGGCATT AAATTGGTTG CAGGCCCTTT
ATCAACTGCC

3001  TGGACCCCAT TTGATCGGAA AGTGGTGCAG TACGCCGGAG
AGATCTACAA

3051  TTATGACTTC CCGGAGTACG GAGCTGGGCA TGCAGGGGCG
TTCGGGGATC

3101  TTCAAGCCAG AACAATCACC AGTAATGACC TGTACGCCAA
CACGAATTTA

3151  GTATTGCAAA GACCCAACAC AGGCACCATC CATGTTCCTT
ACACGCAGGC

3201  ACCGTCAGGC TTTGAGCAGT GGAAGAAAGA CAAACCACCA
TCATTAAAGT

3251  ACACCGCACC ATTTGGGTGC GAAATTCATG TGAATCCCGT
CAGAGCTGAG

3301  AATTGCGCAG TAGGATTTAT ACCATTAGCC TTCGACATAC
CCGATGCCTT

3351  GTTTACCAGG GTGTCAGAAA CACCGACGTT GTCGAGCGCT
GAGTGCTCCT

3401  TGAATGAGTG TACATACTCA ACGGACTTTG GCGGGATCGC
CACTGTCAAG

3451  TACTCGGCTA GTAAATCAGG CAAATGCGCA GTACATGTTC
CCTCAGGCAC

3501  TGCAACTCTG AAAGAGTCAT TGGTGGAAGT GGTCGAACAA
GGGTCAATGA

3551  CCCTTCACTT TTCGACCGCC AGTATACACC CAGAGTTTAA
ATTGCAGATC

3601  TGTACGAAGG TACTCACATG TAAAGGCGAC TGTCATCCGC
CTAAAGACCA
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84

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   3651  TATTGTGACG CACCCCCAGC ACCACGCCCA GACATTTACA
GCTGCGGTAT

   3701  CCAAGACCGC TTGGACGTGG TTAACGTCAC TCTTAGGAGG
GTCAGCAGTA

   3751  ATTATTATAA TTGGCCTTGT ATTAGCAACT GTTGTTGCTA
TGTATGTGCT

   3801  GACCAACCAG AAACATAATT AGACCACAGC AGCGATTGGA
AAGCTGCCTA

   3851  TTAGAAACAT GTAGCGGCAA TTGGCAAGCC GCCTATAAAT
GTTTAGCAGC

   3901  AATTGGCAAG CTGCATATAT AAATTACCTA GCGGCAATTG
GCACGCCGCT

   3951  TATAAAATTT TTATTTTCTT TTACCAATAA TTGGATTTTG
TTTTTAATAT

   4001  TTC
```

85

What is claimed is:

    1. A DNA comprising an isolated and purified western equine encephalitis (WEE) virus cDNA fragment

5   coding for infectious western equine Encephalitis virus genome.

    2. A DNA according to claim 1 wherein said WEE cDNA fragment is operably linked to a promoter

10 such that said cDNA is transcribed.

    3. The DNA according to claim 1, wherein said cDNA fragment contains a deletion in the E3-E2 cleavage site.

15

    4. The DNA according to claim 3, wherein said cDNA fragment deletion is 5 amino acids at the E2-E2 cleavage site said amino acids being Arg Arg Pro Lys Arg.

20

    5. The DNA according to claim 4 wherein said cDNA fragment further contains a suppressor mutation.

    6. The DNA according to claim 5 wherein said

25 suppressor mutation is a substitution of glutamic acid at codon 182 of E2 to lysine.

    7. The DNA according to claim 5 wherein said suppressor mutation is a substitution of glutamic acid

30 at codon 181 of E2 to lysine.

    8. The DNA according to claim 2 wherein said promoter is T7 promoter of pBluescript KS and said DNA is pWE2000.

9. An infectious WEE virus RNA transcript
encoded by the cDNA fragment of claim 1.

5   10. A DNA according to claim 5 wherein said
WEE cDNA fragment is operably linked to a promoter
such that said cDNA is transcribed.

11. An attenuated WEE virus RNA transcript
10   encoded by the cDNA fragment of claim 5.

12. A DNA according to claim 6 wherein said
WEE cDNA fragment is operably linked to a promoter
such that said cDNA is transcribed.
15

13. An attenuated WEE virus RNA transcript
encoded by the cDNA fragment of claim 6.

14. A DNA according to claim 7 wherein said
20   WEE cDNA fragment is operably linked to a promoter
such that said cDNA is transcribed.

15. An attenuated WEE virus RNA transcript
encoded by the cDNA fragment of claim 7.
25

16. Infectious WEE virus particles
containing an RNA transcript according to claim 9.

17. Attenuated WEE virus particles
30   containing an RNA transcript according to claim 11.

18. Attenuated WEE virus particles
containing an RNA transcript according to claim 13.

87

19. Attenuated WEE virus particles
containing an RNA transcript according to claim 15.

20. A live attenuated western equine
5   encephalitis (WEE) virus vaccine comprising attenuated
western equine encephalitis virus according to claim
17.

21. A live attenuated western equine
10   encephalitis (WEE) virus vaccine comprising attenuated
Western Equine Encephalitis virus according to claim
18.

22. A live attenuated western equine
15   encephalitis (WEE) virus vaccine comprising attenuated
western equine encephalitis virus according to claim
19.

23. A DNA comprising an isolated and
20   purified venezuelan equine encephalitis virus IE
variant (VEE IE) cDNA fragment coding for infectious
venezuelan equine encephalitis virus IE variant virus
genome.

25   24. A DNA according to claim 23, wherein
said cDNA fragment has the sequence of SEQ ID NO:2 or
a portion thereof, or an allelic portion thereof.

25. The DNA according to claim 23, wherein
30   said cDNA fragment contains a deletion in the E3-E2
cleavage site.

26. A DNA according to claim 25, wherein
said cDNA fragment contains a deletion according to

claim 25 wherein said deletion is four amino acids
said amino acids being Arg Gly Lys Arg.

27. A DNA fragment according to claim 26
5   wherein said VEE IE cDNA further contains a suppressor
mutation.

28. A DNA according to claim 23 wherein said
VEE IE cDNA fragment is operably linked to a promoter
10   such that said cDNA is transcribed.

29. An infectious VEE IE virus RNA
transcript encoded by the cDNA fragment of claim 23.

15   30. A DNA according to claim 27 wherein said
VEE IE cDNA fragment is operably linked to a promoter
such that said cDNA is transcribed.

31. An attenuated VEE IE virus RNA
20   transcript encoded by the cDNA fragment of claim 27.

32. Infectious VEE IE virus particles
containing an RNA transcript according to claim 29.

25   33. Attenuated VEE IE virus particles
containing an RNA transcript according to claim 31.

34. A live attenuated venezuelan equine
encephalitis virus IE variant (VEE IE) virus vaccine
30   comprising attenuated venezuelan equine encephalitis
virus IE variant virus according to claim 33.

35. A bivalent alphavirus vaccine comprising
live attenuated western equine encephalitis (WEE)
35   virus comprising an attenuating mutation and live

attenuated venezuelan equine encephalitis virus IE
variant (VEE IE) comprising an attenuating mutation.

5   36. A bivalent alphavirus vaccine according
to claim 35 wherein said live attenuated western
equine encephalitis virus is chosen from the group
consisting of attenuated WEE comprising a deletion in
the E3-E2 cleavage site and a substitution of glutamic
acid at codon 182 of E2 to lysine, and attenuated WEE
10   comprising a deletion in the E3-E2 cleavage site and a
substitution of glutamic acid at codon 181 of E2 to
lysine.

       37. An attenuated western equine
15   encephalitis virus wherein said virus comprises an
attenuating mutation selected from the group
consisting of: a C to T change at nucleotide 7 of the
5' noncoding region of the WEE genome, a A to G change
at nucleotide 13 of the 5' noncoding region of the WEE
20   genome, a T to A change at nucleotide 25 of the 5'
noncoding region of the WEE genome, and a deletion of
an A at nucleotide 22 of the 5' noncoding region of
the WEE genome.

25   38. A pharmaceutical formulation comprising
attenuated WEE virus particles according to claim 17
in an effective immunogenic amount in a
pharmaceutically acceptable carrier.

30   39. A pharmaceutical formulation comprising
attenuated WEE virus particles according to claim 18
in an effective immunogenic amount in a
pharmaceutically acceptable carrier.

40. A pharmaceutical formulation comprising
attenuated WEE virus particles according to claim 19
in an effective immunogenic amount in a
pharmaceutically acceptable carrier.

5

41. A pharmaceutical formulation comprising
attenuated VEE IE virus particles according to claim
33 in an effective immunogenic amount in a
pharmaceutically acceptable carrier

10

42. An attenuated chimeric virus comprising
nonstructural sequences from a first alphavirus and
structural sequences from a second alphavirus
resulting in attenuation of the second alphavirus.

15

43. An attenuated chimeric virus according
to claim 42 wherein wherein said first alphavirus is
western equine encephalitis virus and said second
alphavirus is chosen from the group consisting of:
20   Sindbis virus, Aura virus, Barmah Forest virus, Bebaru
Cabassou virus, Chikungunya virus, Everglades virus,
Fort Morgan virus, Getah virus, Highlands J virus,
Kyzylagach virus, Mayaro virus, Middelburg virus,
Mucambo virus, Ndumu virus, O'nyong-nyong virus,
25   Pixuna virus, Ross River virus, Sagiyama virus,
Semliki Forest virus, SAAR87 virus, Tonate virus, Una
virus, venezuelan equine encephalitis virus, and
Whataroa virus.

30   44. An attenuated second virus according to
claim 43 wherein said second alphavirus is eastern
equine encephalitis virus.

45. An attenuated chimeric second virus
35   according to claim 42 wherein said first alphavirus is

Venezuelan equine encephalitis virus variant IE and
said second alphavirus is chosen from the group
consisting of: venezuelan equine encephalitis virus,
western eqine encephalitis virus, eastern equine
5   encephalitis virus, Sindbis virus, Aura virus, Barmah
Forest virus, Bebaru Cabassou virus, Chikungunya
virus, Everglades virus, Fort Morgan virus, Getah
virus, Highlands J virus, Kyzylagach virus, Mayaro
virus, Middelburg virus, Mucambo virus, Ndumu virus,
10   O'nyong-nyong virus, Pixuna virus, Ross River virus,
Sagiyama virus, Semliki Forest virus, SAAR87 virus,
Tonate virus, Una virusand Whataroa virus.

46. An attenuated chimeric second virus
15   according to claim 44 wherein said second virus is
venezuelan equine encephalitis virus variant IA.

47. An attenuated chimeric second virus
according to claim 42 wherein said first alphavirus is
20   venezuelan equine encephalitis virus variant IA and
said second alphavirus is chosen from the group
consisting of: Venezuelan equine encephalitis virus,
western eqine encephalitis virus, eastern equine
encephalitis virus, Sindbis virus, Aura virus, Barmah
25   Forest virus, Bebaru Cabassou virus, Chikungunya
virus, Everglades virus, Fort Morgan virus, Getah
virus, Highlands J virus, Kyzylagach virus, Mayaro
virus, Middelburg virus, Mucambo virus, Ndumu virus,
O'nyong-nyong virus, Pixuna virus, Ross River virus,
30   Sagiyama virus, Semliki Forest virus, SAAR87 virus,
Tonate virus, Una virus, and Whataroa virus.

48. An attenuated chimeric second virus
according to claim 47 wherein said second virus is
35   venezuelan equine encephalitis virus variant IE.

49. An attenuated chimeric second virus according to claim 47 wherein said second virus is Venezuelan Equine Encephalitis virus variant IIIA.

50. An attenuated virus vaccine comprising chimeric virus according to claim 42 wherein said vaccine is directed against said second alphavirus.

51. An inactivated western equine encephalitis virus (WEE) vaccine comprising attenuated WEE according to claim 17 wherein said attenuated WEE is inactivated.

52. An inactivated western equine encephalitis virus (WEE) vaccine comprising attenuated WEE according to claim 18 wherein said attenuated WEE is inactivated.

53. An inactivated western equine encephalitis virus (WEE) vaccine comprising attenuated WEE according to claim 19 wherein said attenuated WEE is further inactivated.

54. An inactivated venezuelan equine encephalitis virus variant IE (VEE IE) vaccine comprising attenuated VEE IE according to claim 33 wherein said attenuated VEE IE is further inactivated.

55. An inactivated eastern equine encephalitis virus (EEE) vaccine comprising attenuated EEE according to claim 44 wherein said attenuated virus is further inactivated.

93

56. An inactivated venezuelan equine encephalitis virus variant IA (VEE/IA) vaccine comprising attenuated VEE/IA according to claim 46 wherein said attenuated virus is further inactivated.

5

57. An inactivated venezuelan equine encephalitis virus variant IIIA (VEE/IA) vaccine comprising attenuated VEE IIIA according to claim 49 wherein said attenuated virus is further inactivated.

10

58. An inactivated venezuelan equine encephalitis virus variant IE (VEE IE) vaccine comprising attenuated VEE IE according to claim 48 wherein said attenuated virus is further inactivated.

15

59. A method for expressing a protein said method comprising cloning a gene encoding said protein into a an attenuated virus replicon, said replicon chosen from the group consisting essentially of
20   attenuated WEE and attenuated VEE IE wherein transcription of said replicon yields RNA capable of infecting a cell in which said protein is to be expressed.

25   60. A method for the diagnosis of western equine encephalitis virus (WEE) infection comprising the steps of :
(i) contacting a sample from an individual suspected of having a WEE infection with all or a
30   unique portion of WEE; and
(ii) detecting the presence or absence of a WEE infection by detecting the presence or absence of a complex formed between WEE and antibodies specific therefor in the sample.

61. A method for the diagnosis of venezuelan equine encephalitis virus variant IE (VEE IE) infection comprising the steps of :

5   (i) contacting a sample from an individual suspected of having a VEE IE infection with all or a unique portion of VEE IE; and

        (ii) detecting the presence or absence of a VEE IE infection by detecting the presence or absence

10   of a complex formed between VEE IE and antibodies specific therefor in the sample.

        62. A method for the diagnosis of western equine encephalitis virus (WEE) from a sample using

15   the polymerase chain reaction, said method comprising:

        (i) extracting RNA from the sample;

        (ii) reverse transcribing the RNA of (i) to DNA;

        (iii) contacting said DNA with

            (a) at least four nucleotide triphosphates,

20   (b) a primer that hybridizes to WEE DNA, and

            (c) an enzyme with polynucleotide synthetic activity,

        under conditions suitable for the hybridization and extension of said first primer by said enzyme,

25   whereby a first DNA product is synthesized with said DNA as a template therefor, such that a duplex molecule is formed;

        (iv) denaturing said duplex to release said first DNA product from said DNA;

30   (v) contacting said first DNA product with a reaction mixture comprising:

            (a) at least four nucleotide triphosphates,

            (b) a second primer that hybridizes to said first DNA, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said second primer by said enzyme,

5   whereby a second DNA product is synthesized with said first DNA as a template therefor, such that a duplex molecule is formed;

(vi) denaturing said second DNA product from said first DNA product;

10   (vii) repeating steps iii-vi for a sufficient number of times to achieve linear production of said first and second DNA products;

(viii) fractionating said first and second DNA products generated from said WEE DNA; and

15   (ix) detecting said fractionated products for the presence or absence of WEE in a sample.

63. A method for the diagnosis of venezuelan equine encephalitis variant IE virus (VEE IE) from a

20   sample using the polymerase chain reaction, said method comprising:

(i) extracting RNA from the sample;

(ii) reverse transcribing said RNA of (i) to DNA;

(iii) contacting said DNA with

25   (a) at least four nucleotide triphosphates,

(b) a primer that hybridizes to VEE IE DNA, and

(c) an enzyme with polynucleotide synthetic activity,

30   under conditions suitable for the hybridization and extension of said first primer by said enzyme, whereby a first DNA product is synthesized with said DNA as a template therefor, such that a duplex molecule is formed;

96

(iv) denaturing said duplex to release said first DNA product from said DNA;

(v) contacting said first DNA product with a reaction mixture comprising:

5

(a) at least four nucleotide triphosphates,

(b) a second primer that hybridizes to said first DNA, and

(c) an enzyme with polynucleotide synthetic activity,

10

under conditions suitable for the hybridization and extension of said second primer by said enzyme, whereby a second DNA product is synthesized with said first DNA as a template therefor, such that a duplex molecule is formed;

15

(vi) denaturing said second DNA product from said first DNA product;

(vii) repeating steps iii-vi for a sufficient number of times to achieve linear production of said first and second DNA products;

20

(viii) fractionating said first and second DNA products generated from said VEE IE DNA; and

(ix) detecting said fractionated products for the presence or absence of VEE IE in a sample.

25

64. A method for providing protective immunity against a second alphavirus to individuals with pre-existing immunity to a first alphavirus said method comprising administering to said individuals an effective amount of live attenuated second alphavirus.

30

65. A method for providing protective immunity against a second alphavirus according to claim 64, wherein said live attenuated second alphavirus is attenuated western equine encephalitis

35 virus.

66. The method of claim 65 wherein said western equine encephalitis (WEE) virus is chosen from the group consisting of: attenuated WEE having a

5   substitution of glutamic acid at codon 181 of E2 to lysine, attenuated WEE having a substitution of glutamic acid at codon 181 of E2 to lysine, attenuated WEE having a C to T change at nucleotide 7 of the 5' noncoding region of the WEE genome,

10   attenuated WEE having a A to G change at nucleotide 13 of the 5' noncoding region of the WEE genome, attenuated WEE having a T to A change at nucleotide 25 of the 5' noncoding region of the WEE genome, and attenuated WEE having a deletion of an A at nucleotide

15   22 of the 5' noncoding region of the WEE genome.

67. A method for providing protective immunity against a second alphavirus according to claim 64, wherein said live attenuated second

20   alphavirus is attenuated venezuelan equine encephalitis virus variant IE.

68. A WEE infection diagnostic kit comprising primers specific for WEE and ancillary

25   reagents suitable for use in detecting the presence or absence of WEE in a mammalian sample.

69. A VEE IE infection diagnostic kit comprising primers specific for VEE IE and ancillary

30   reagents suitable for use in detecting the presence or absence of VEE IE in a mammalian sample.

70. An isolated and purified Venezuelan equine encephalitis variant IIIA cDNA encoding the VEE

35   IIIA structural genes.

71. An amino acid fragment encoded by the
cDNA fragment according to claim 70.

5   72. A polypeptide encoded by the amino acid
fragment according to claim 71.

10

15

20

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35

Asssembly mₒ ₋ule WE3'-17   1 / 7
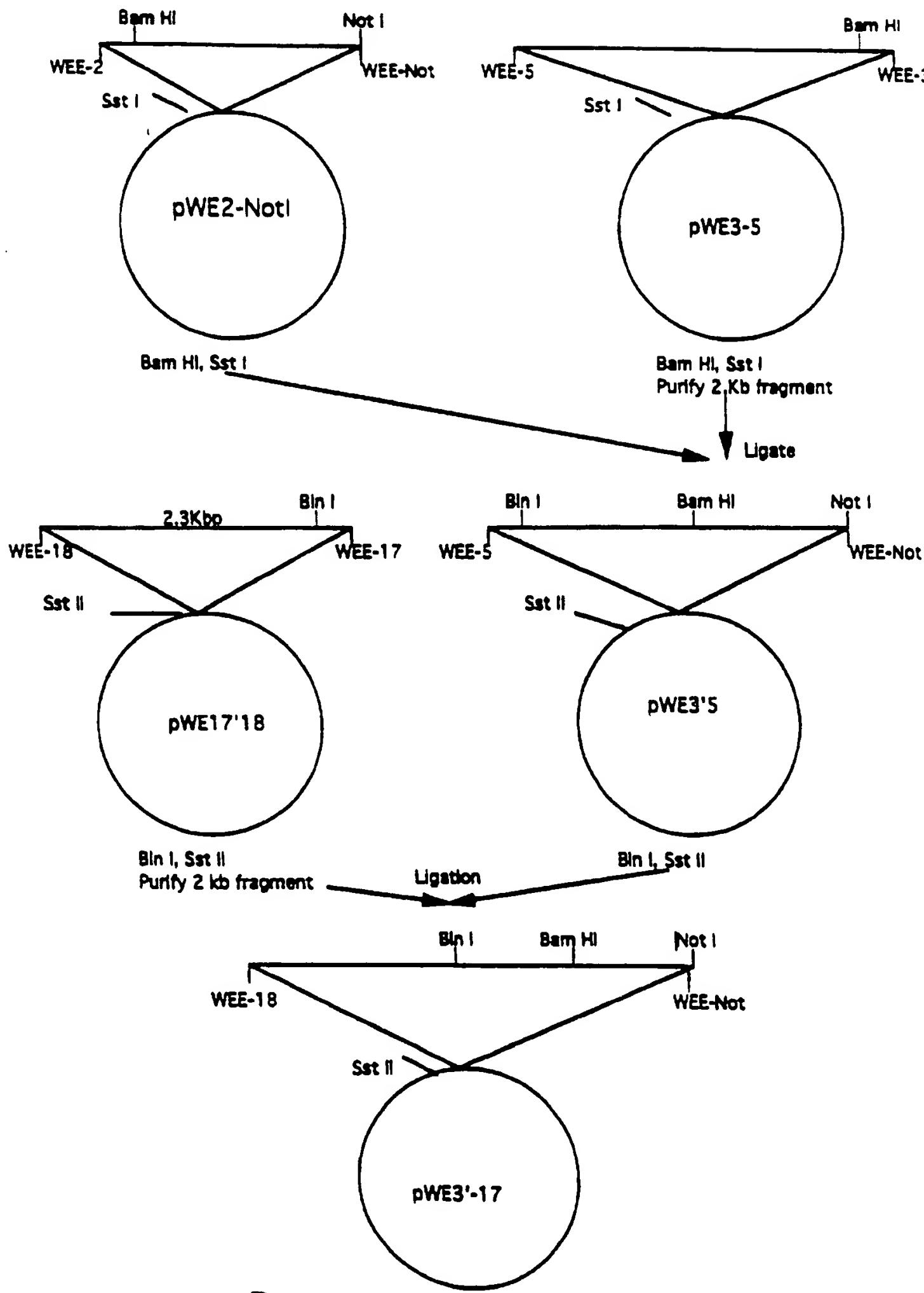
FIGURE 1A

Assembly c noc J pWE5'-18   2 / 7
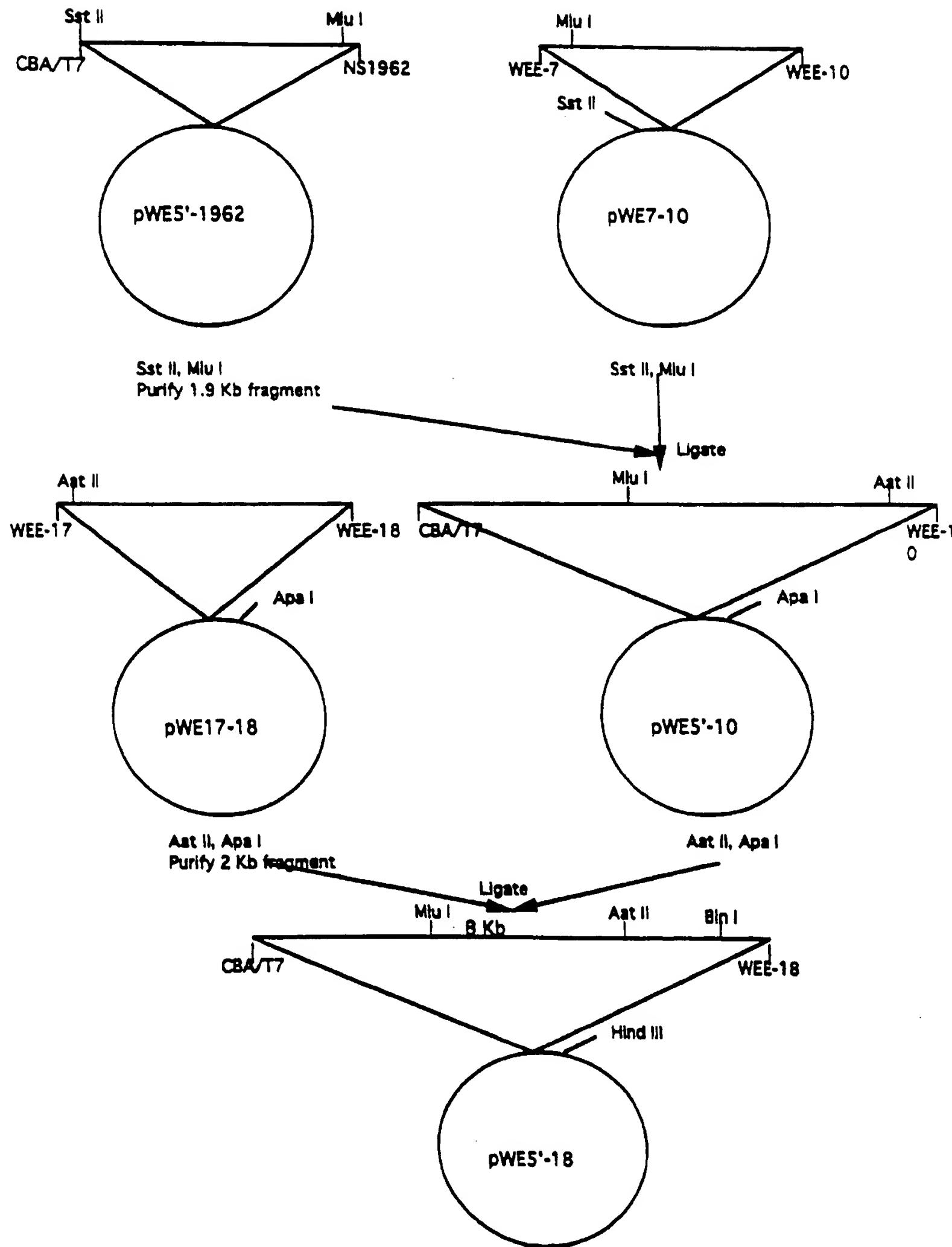
FIGURE 1B

Assembly ⊾ ⌐WE∠000 full length cDNA clone of Western
equine encephalitis virus from modules pWE5'-18 and
pWE3'-17
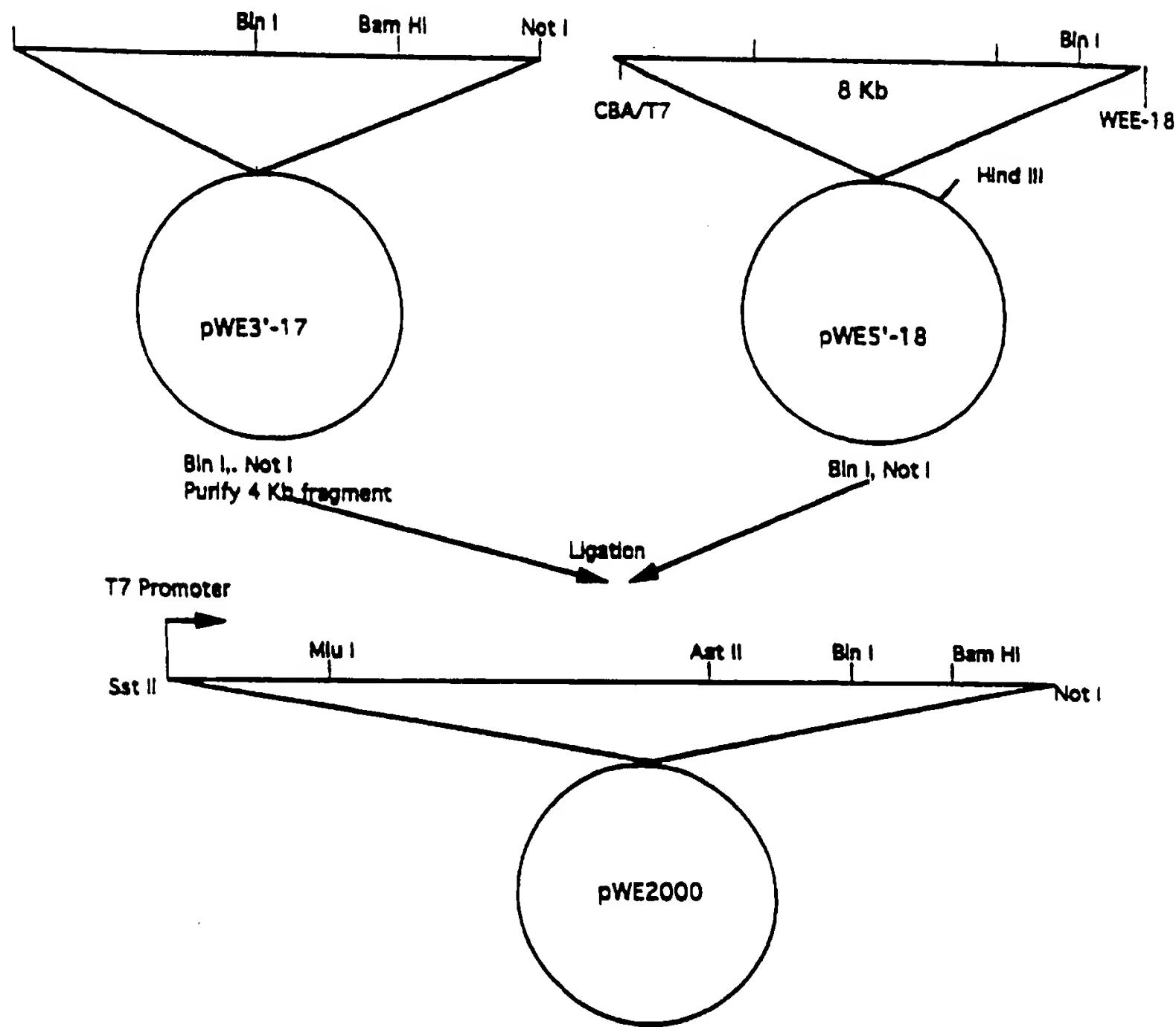
FIGURE 1C
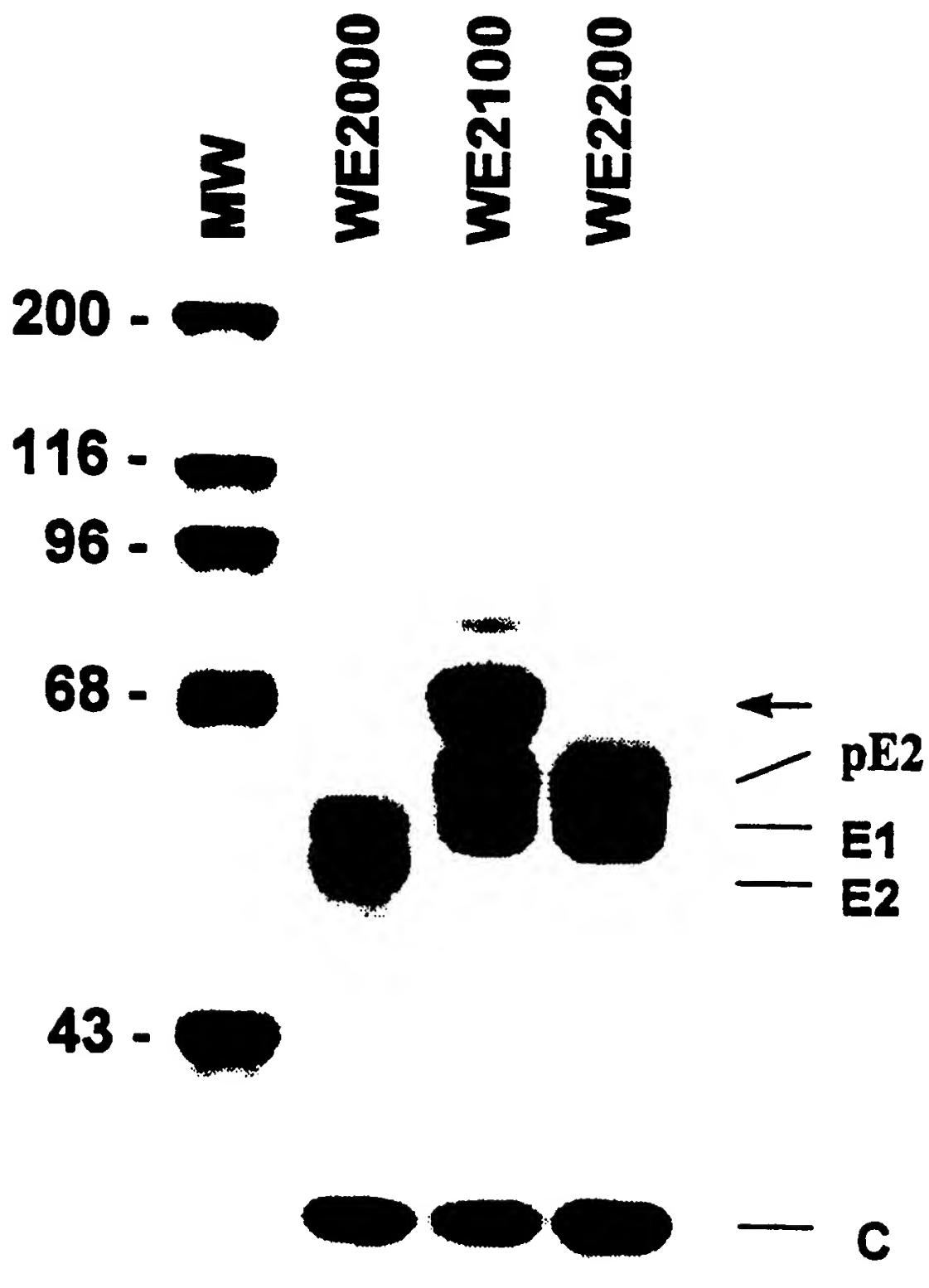
FIGURE 2
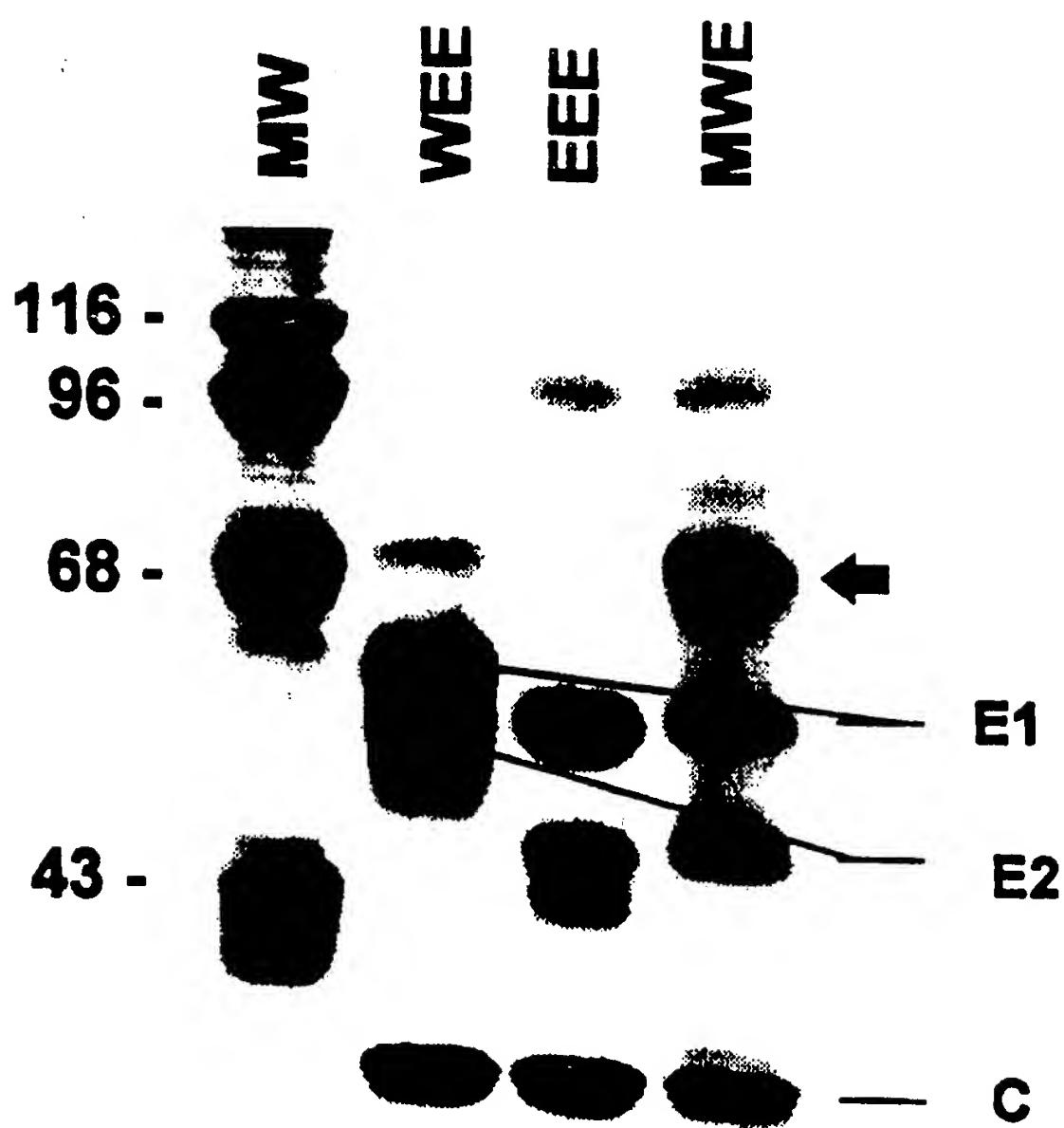
FIGURE 3

Figure 4
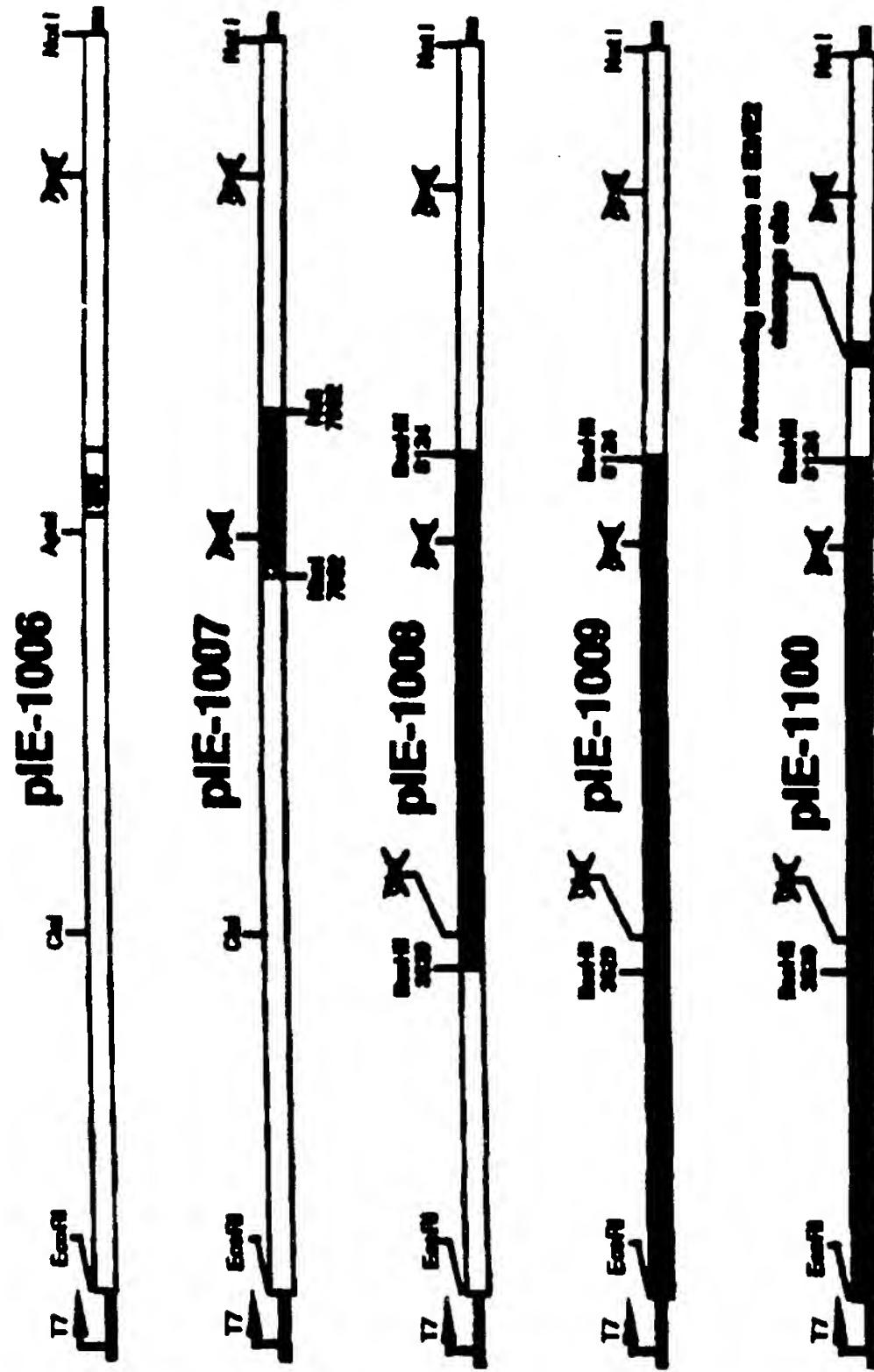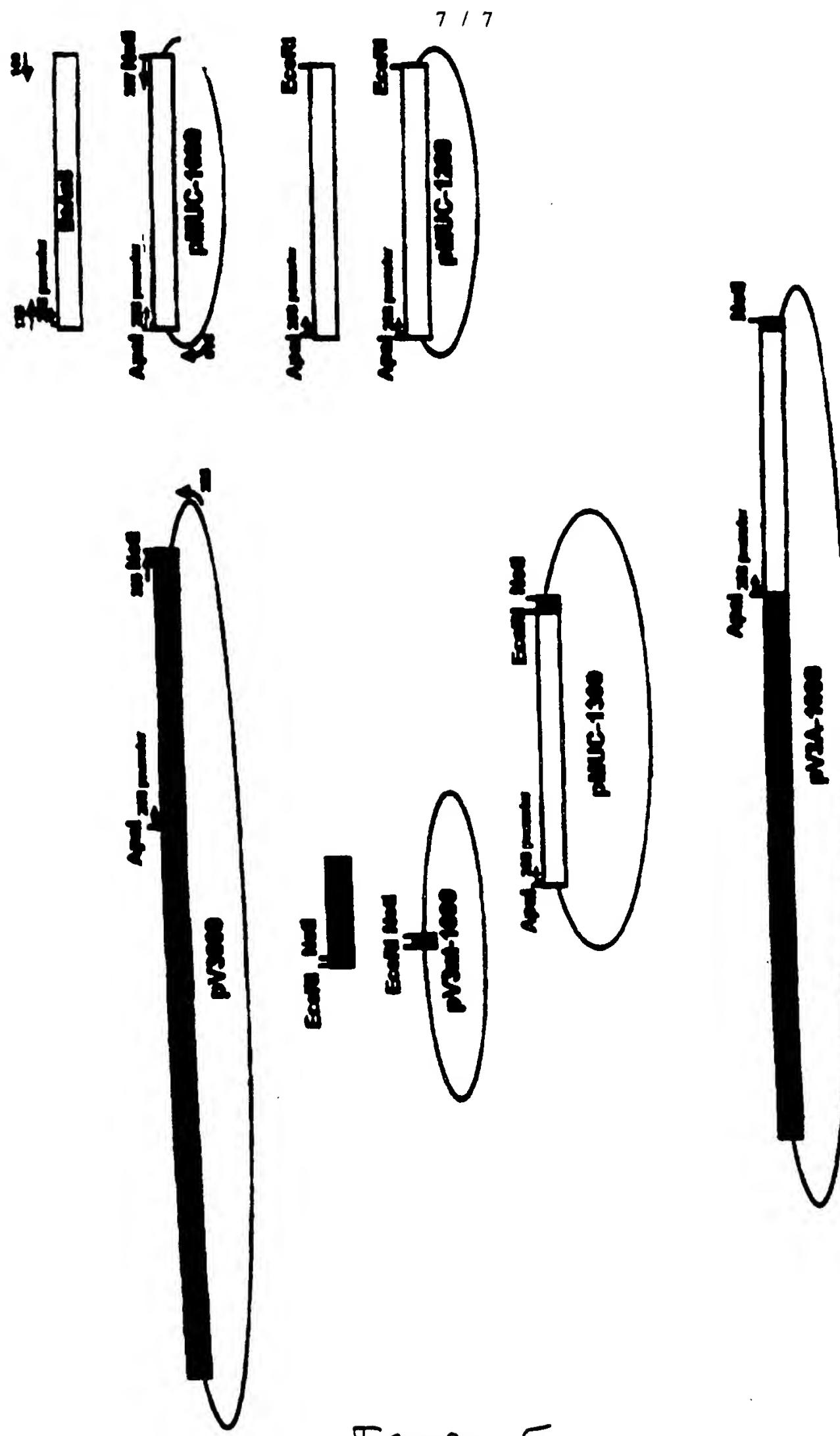
FIGURE 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/10645

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :C12N 15/40, 7/01, 7/04, 15/86; A61K 39/193.
US CL   :536/23.72; 435/235.1, 236, 69.1, 172.3; 424/205.1, 218.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   536/23.72; 435/235.1, 236, 69.1, 172.3; 424/205.1, 218.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Aps, Biosis, Cab, Derwent WPI. Search terms: western, equine(w)enceph?, attenuat?, venezuelan, genom?, ma. clon?, sequenc?, dna, dnas, "E2", "E3", wee.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FRAIZER, G. et al. Isolation and preliminary characterization of mutants of Western equine encephalomyelitis virus with altered virulence in chickens. Biological Abstracts. 1985. Vol. 80, No. 5, page AB-506, Abstract no. 41179, see entire abstract.	16
Y	US 5,185,440 A (DAVIS et al) 09 February 1993, see entire document, particularly column 7, line 66 through column 8, line 1.	1-3, 8, 9, 16, 59
Y	US 5,505,947 A (JOHNSTON et al) 09 April 1996, see particularly column 3, lines 50-54, Abstract, and Examples 1 and 2.	3, 59

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

Date of the actual completion of the international search	Date of mailing of the international search report
27 AUGUST 1998	06 OCT 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	MARY E. MOSHER, PH.D.
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)☆

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WEAVER, S.C. et al. A comparison of the Nucleotide Sequences of Eastern and Western Equine Encephalomyelitis Viruses with Those of Other Alphaviruses and Related RNA Viruses. Virology. 1993. Vol. 197, pages 375-390. See figures 3 and 4.	1-3, 8, 9, 16, 59
Y	HAHN, C.S. et al. Western equine encephalitis virus is a recombinant virus. Proceedings of the National Academy of Sciences USA. August 1988. Vol. 85, p. 5997-6001. See Figure 1.	1-3, 8, 9, 16, 59
A	STRAUSS, J.H. et al. The Alphaviruses: Gene Expression, Replication, and Evolution. Microbiological Reviews. September 1994. Vol. 58, No. 3, pages 491-562.	1-22, 37-40, 51-53, 59

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/10645

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such
   an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable
   claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers
   only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-22, 37-40, 51-53, 59

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.
                         ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)☆

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group 1, claims 1-22, 37-40, 51-53, 59, drawn to a first product DNA encoding an infectious genome of Western Equine Encephalitis (WEE) virus, RNA and virus encoded by DNA, vaccines and pharmaceuticals comprising virus encoded, and methods of use.

Group 2, claims 23-34, 41, 54, 59 drawn to second product DNA encoding an infectious genome of Venezuelan Equine Encephalitis IE variant (VEE IE) virus, RNA and virus encoded by DNA, vaccines and pharmaceuticals comprising virus encoded, and methods of use.

Group 3, claims 35-36, drawn to third product, combination vaccine comprising attenuated WEE and attenuated VEE IE

Group 4, claims 42-50, 55-58, drawn to fourth product, chimeric alphavirus and vaccine.

Group 5, claims 62, 68, drawn to fifth product, WEE specific primer kit, and method of use.

Group 6, claims 63, 69, drawn to sixth product, VEE IE specific primer kit, and method of use

Group 7, claims 70-72 , drawn to seventh product, DNA encoding structural genes of VEE variant IIIA and products encoded.

Group 8, claims 60 , drawn to method of diagnosing WEE using antigen.

Group 9, claims 61, drawn to method of diagnosing VEE IE using antigen.

Group 10, claims 64-67, drawn to method of vaccinating immune individual using a second alphavirus.

The inventions listed as Groups 1-10 do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

In groups 1 and 2, the special technical feature in each case is the DNA encoding an infectious genome of a particular virus; since groups 1 and 2 pertain to two distinct viruses, they involve different special technical features.

Group 3 does not have the corresponding special technical feature, because the attenuated viruses required in group 3 are not limited to those made by using the DNAs of groups 1 or 2; for example claim 35 encompasses combination vaccines using viruses attenuated by propagation in culture.

Group 4 does not have the corresponding special technical feature, because it is not limited to either of the viruses encoded by the DNAs of group 1 or group 2; for example claim 42 encompasses a chimera made from Sindbis and Semliki Forest viruses.

Groups 5 and 6 do not have the corresponding special technical feature, because they require small, specific primers, not infectious full-length genomic materials.

Group 7 does not have the corresponding special technical feature, because is drawn to a sub-genomic fragment of a third virus.

Groups 8 and 9 do not have the corresponding special technical feature, because they are not limited to materials of groups 1 or 2; for example claim 60 encompasses a method using viral antigens produced by propagation of native WEE in standard tissue culture.

Group 10 does not have the corresponding special technical feature because it is not limited to the viruses made using the DNAs of groups 1 or 2; for example claim 64 encompasses a method of vaccinating a Sindbis-immune monkey

against Semliki Forest virus.